5-MTP inhibits cancer-derived succinate-mediated oncogenic responses

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Endothelium-derived 5-methoxytryptophn (5-MTP) has been identified as a tryptophan metabolite with anti-tumor and anti-inflammatory properties. Its effect on cancer-derived secreted metabolites involved in cancer progression is of value for further investigation. Succinate secreted by cancer cells has been identified as an oncometabolite that triggers the polarization of macrophages into tumor-associated macrophages (TAMs) and promotes cancer metastasis. Our results revealed that 5-MTP reduced lung cancer A549 succinate secretion level compared with vehicle and suppressed succinate-mediated TAM polarization. In addition, 5-MTP inhibited succinate-driven cancer metastasis by inhibiting succinate/succinate receptor-mediated oncogenic signaling. Conversely, succinate reduced 5-MTP production and secretion of human aortic endothelial cells (HAECs) which was accompanied by increasing HAEC permeability. These results provide new insight into the physiological-oncogenic interaction between the endothelium-derived protective metabolite 5-MTP and the cancer-secretory oncometabolite succinate in the host oncogenic defense system.

A chemogenetic approach reveals a GPCR-Gas-PKA signaling axis promoting T cell dysfunction and cancer immunotherapy failure

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Recent advances in immune checkpoint blockade (ICB) inhibiting programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein (CTLA-4) have revolutionized the standard of

care for cancer treatment. However, the limited response rates to ICB across multiple cancer types suggest that new approaches and targets are clearly needed in order to achieve durable responses (cure). G protein-coupled receptors (GPCRs) are the most intensively studied drug targets, primarily due to their druggability and relevance to most physiological processes and disease conditions. Here, we applied a new computational pipeline to cross-integrate hundreds of thousands of CD8 T cells from multiple single cell RNA-seq datasets from 13 distinct cancer types, uncovering a significant enrichment of Gas-coupled GPCRs on exhausted T cells. These include EP2, EP4, A2AR, b1AR, and b2AR, all of which promote T cell dysfunction by inhibiting cytotoxicity and cytokine secretion. Using a novel synthetic biology approach, we developed a chemogenetic CD8-restricted Gas-DREADD (Designer Receptor Exclusively Activated by A Designer Drug) transgenic mouse model in which activation of Gas signaling is temporally and spatially controlled. Utilizing this Gas-DREADD model, we discovered that the Gas-signaling axis represents a previously uncharacterized signaling axis that dampens the anti-tumor CD8 T cell activity and leads to ICB immunotherapy failure. Our findings reveal that Gas-coupled GPCRs may represent new targetable immune checkpoints that can be combined with ICB as part of novel multimodal precision approaches to enhance the response to immunotherapies.

A functional genomics approach to understand the biology of human tumor associated macrophages in vivo

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Mice repopulated with a human immune system (“humanized mice”) represent an invaluable tool in that they provide a tractable model of human tumor progression with the additional context of fully differentiated human immune cells that interact with the tumor *in vivo*. We are using the MISTRG strain as recipient mice, which support the multilineage development of a human immune system, including functional B and T lymphocytes, NK cells, myelomonocytic cells and dendritic cells. Humanized MISTRG mice not only support the growth of engrafted human melanomas, but the human macrophages infiltrate the tumor and support its metastatic spread. Tumor associated macrophages in this model exhibit transcriptional profiles distinct from macrophages in the periphery. Furthermore, this tumor-specific macrophage profile is remarkably similar to that seen in patient melanoma samples, suggesting that our humanized mouse model is a faithful representation of the immune cell phenotypes in a human tumor microenvironment. To better understand the biology of tumor-associated macrophages in vivo, we are applying to our model the recently developed “ProCode” CRISPR-based screening tool to

determine the key mechanisms facilitating their differentiation, homing and persistence in the tumor microenvironment in vivo. This approach aims to identify novel targets for immunotherapy-based interventions that would specifically target pathologically-relevant mechanisms, as macrophages in the tumor are known to contribute to tumor progression, but in the periphery remain critical for coordinating immune defenses against pathogens.

A lymph node-to-tumour immunosuppressive macrophage circuit driven by dendritic cell immunotherapy inhibits anti-tumour immunity

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Immune-checkpoint blockers (ICB) provide limited benefit against T cell-depleted tumours. T cell potentiating cellular immunotherapies, like dendritic cell (DC) vaccines, offer great promise against them. Unfortunately, most cancer patients are unresponsive to current DC vaccines. Presently, it is unclear if this is due to limited immunogenicity or unknown immuno-resistance mechanisms. Therefore, we used computational mapping of cancer patients' multi-omics data,

to design a DC vaccine exhibiting type I interferon (IFN)-driven immunogenic maturation-trajectory, whilst integrating immunogenic cancer cell death (ICD). Although this DC vaccine exhibited high immunogenicity and was tailored against a rationally-selected murine T cell-depleted tumour model, yet it could not eliminate these tumours. Unexpectedly, the high immunogenicity of DC vaccines countered their own anti-tumour efficacy i.e., in the lymph nodes (LNs), instead of activating CD4+ / CD8+T cells, the DCs stimulated immunosuppressive PDL1+ LN-associated macrophages (LAMs). Subsequently, DC vaccines also fuelled the pre-existing, T cell-suppressive, PDL1+ tumour□associated macrophages (TAMs). This created a CD4+ /CD8+T cell-suppressive circuit of PDL1+macrophages, spanning across LNs and tumours. Combining DC vaccines with anti-PDL1 ICB, depleted PDL1+ LAMs/TAMs, suppressed LN/tumoural myeloid inflammation, and unleashed effector and effector/stem-like memory T cells, causing tumour regression. Interestingly, clinical DC vaccines also potentiated lymphocyte-suppressive PDL1+TAMs in patients with prototypical T cell-depleted tumours. This work illustrates a novel LAMs/TAMs-driven immuno-suppressive circuit, induced by DC□immunotherapy, which antagonises its efficacy against T cell-depleted tumours. This opens exciting avenues for combinatorial immunotherapy.

A Novel Platform to Enrich for Tumor-Specific T Cells in Non-Small Cell Lung Cancer

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To induce robust cellular immunity against non-small cell lung cancer (NSCLC), the leading cause of cancer-related death in the US, we have developed an innovative cell-based therapeutic platform technology to generate polyclonal, neoantigen-reactive T cells *in vitro*. Surgically resected NSCLC tissue and peripheral matched blood were collected from 17 patients under an IRB-approved protocol. From 3 of the patient-derived NSCLC specimens, methods were tested to enhance immunogenicity and stimulate neoantigen-specific immune responses. For these patients, the therapeutic platform induced a significant increase in T cell proliferation, interferon-gamma secretion, and blast phenotype of lymphocyte populations compared to T cells primed by standard methods. Interestingly, for one patient, immunosuppression of the T

cell response with concomitant increase in CD96, an immune checkpoint receptor implicated in lung cancer progression, was observed in the tumor. Importantly, using sequencing data from one patient, an analytical and machine learning pipeline detected putative neoantigens using our therapeutic platform technology and not with standard methods. The therapeutic platform can be used to directly generate cell-based immunotherapies and to facilitate neoantigen discovery and development of targeted cancer immunotherapies. We then use a novel, human vascularized microtumor (VMT) platform to assess the sensitivity of tumor cells to T-cell mediated attack at the individual patient level. Our goal is to advance novel approaches to effectively mobilize anti-tumor immunity and overcome treatment resistance in NSCLC.

A PD-L1 reporter cell line based on the immune checkpoint protein profiling of ATCC cell lines facilitates cancer immunotherapy drug screening

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The success of immune checkpoint inhibitors in the treatment of various types of cancers and their continued growth in the market have driven burgeoning interests in developing more drugs in this category. However, the intrinsic complexity of the immunological models and the variable drug responses among different cancer types have become the most prominent challenges. To facilitate large scale research projects and drug discovery of immune checkpoint inhibitors, we conducted a comprehensive protein profiling of ATCC's vast portfolio of human tumor and immune cell lines for several established and novel immune checkpoint molecules. Based on this protein profiling data, we generated an immune checkpoint reporter cancer cell line with high expression of endogenous programmed death-ligand 1 (PD-L1), a highly validated target for immune checkpoint inhibitor therapeutics. The reporter system contains a gamma interferon activation site (GAS)-response element upstream of the luciferase gene, preventing luciferase expression when PD-L1 binds to programmed death-1 (PD-1) that suppresses T cell-mediated antitumor activity. In the presence of a PD-1/PD-L1 inhibitor, a luciferase expression based bioluminescent signal is produced, which can be readily detected and quantitated to evaluate the efficacy, potency, and dynamics of the inhibitor. Our data showed that the bioluminescence in the reporter cancer cells increased approximately 250 folds in a dose-dependent manner in response to interferon gamma (IFN-γ) stimulation, which mimics the signaling from activated CD8+ cytotoxic T cells. The bioluminescence increased approximately 100 folds in response to CD8+ primary T cell-conditioned media stimulation, and up to 5 folds in

response to co-culture with CD8+ primary T cells in the presence of an anti-PD-L1 blocking antibody in a dose-dependent manner. The luciferase expression and endogenous PD-L1 expression were well maintained after the cell line had reached >30 population doubling level. These results highlight the robustness and responsiveness of the reporter system for the assessment of T cell-mediated immune responses triggered by PD-1/PD-L1 checkpoint inhibitors. This PD-L1 immune checkpoint reporter cancer cell line yields exceptional in vitro, and ex vivo assay sensitivity and reproducibility, while simplifies the complex immunological model by providing physiologically relevant expression of PD-L1, in comparison to similar assays on the market with an artificial PD-L1 overexpression system.

A SREBF2 Gene Program Drives an Immunotolerant Dendritic Cell Population During Cancer Progression

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Conventional dendritic cells (cDCs) are essential mediators of anti-tumor immunity and the efficacy of checkpoint blockade immunotherapies. Because of their importance, cancers have developed mechanisms to render DCs dysfunctional leading to an inability of DCs to activate adaptive immunity. Indeed, studies have demonstrated that dysfunctional DCs drive immune tolerance and can support tumor progression. Alterations in DC metabolism within the tumor microenvironment (TME) play a key role in dictating DC functionality. Within the TME, nutrient deficiency, metabolic by-products, and the release of immunosuppressive factors have profound effects on DC metabolism and function. While immune stimulating DCs rely on glycolysis for their energy needs, tolerogenic DCs have enhanced fatty acid oxidation (FAO) while displaying an accumulation of lipids and increased lipid peroxidation. Under healthy conditions, lipid homeostasis is maintained through a network of transcription factors including sterol-regulatory element-binding protein 2 (SREBP2). Under low sterol conditions, SREBP2 promotes the expression of mevalonate pathway genes and the low-density lipoprotein receptor to both synthesize and import cholesterol. Despite significant advances in DC biology, the role of SREBP2 and the surrounding pathways regulating lipid homeostasis in tolerogenic DCs during tumor progression are poorly understood. In this study, we use single-cell RNA-seq (scRNAseq) and scATAC-seq of DCs isolated from the tumor-draining lymph nodes (TDLN) of transgenic

melanoma mice to interrogate transcriptional and chromatin landscape differences amongst different DC sub-populations. We observed a DC subset enriched in the expression of immunoregulatory genes and maturation markers (mregDCs). Adoptive transfer of DCs into tumor-bearing mice reveals that these DCs migrate from the tumor bed to TDLN tissues during tumor progression and derives primarily from cDC2s. We identified the tetraspanin CD63 as a surface marker to distinguish and sort these mregDCs for downstream analysis. Co-cultures of DCs with T cells demonstrate that $CD63^+$ mregDCs can suppress $CD8^+$ T cell activation via the inhibition of DC antigen cross-presentation while promoting $CD4^+$ T_H2 and $CD4^+FOXP3^+$ regulatory T cell (Tregs) differentiation. $CD63^+$ mregDCs are enriched in cholesterol homeostasis genes driven by SREBP2 and metabolic analysis demonstrates that $CD63^+$mregDCs are more dependent on FAO than other cDC subsets. Importantly, in models of melanoma, CD11c-targeted SREBP2 knockout mice and SREBP2 inhibitor-treated mice exhibit a significant reduction in $CD63^+$ mregDCs and Tregs within TDLN tissues correlating with diminished tumor growth. Furthermore, scRNAseq and spatial transcriptional analyses of sentinel LNs from melanoma patients revealed that the $CD63^+$ mregDC population is conserved in humans and that these DCs are enriched in genes involved in cholesterol homeostasis. Collectively, this work demonstrates an important role of SREBP2 in driving a tolerogenic genetic program in mregDCs and highlights the therapeutic targeting of SREBP2 and DC lipid metabolism as a promising approach to overcome immune tolerance in the TME and enhance immunotherapy responses.

A tumor metabolite impacting immunotherapy efficacy: Cholesterol sulfate regulates tumor-immune interactions

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Tumors constitute an ecosystem of heterogeneous metabolic environments where a variety of metabolites function as intra- and intercellular mediators that affect tumor development. We previously identified that cholesterol sulfate (CS) is an endogenous inhibitor of DOCK2, a Rac activator essential for leukocyte migration and activation. Thereby, local CS production creates immune-evasive/immunosuppressive microenvironments. Many types of human cancers express the SULT2B1 gene, encoding the steroid sulfotransferase SULT2B1b responsible for CS production, and in certain cancers, the gene expression associates with poor prognosis. In colon cancers, level of CS is higher in tumor tissues, and tumor regions with high CS were poorly infiltrated with CD8+ T cells. In syngeneic mouse models, CS-producing cancer cells formed

larger tumors in a host-immunity-dependent manner, and exhibited resistance to immunotherapies via antigen-specific T cell transfer and immune-checkpoint blockade. A novel SULT2B1b inhibitor we identified counteracted the above phenotypes. Thus, cancer-derived CS is a key mediator of tumor-immune interactions, and CS/SULT2B1b may be a potential target for enhancing the efficacy of immunotherapies.

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Aak1 Inhibition Diminishes Efficacy of Anti-PD-1 Immunotherapy in Murine Tumor Models

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Anti-PD-1 immunotherapies are approved for the treatment of a variety of cancers, and their widespread clinical application has revealed gaps in our understanding mechanisms of response or resistance. Response to immune checkpoint blockade (ICB), including anti-PD-1, is variable, and defining response prospectively based on tumor size in patients is difficult as increased tumor sizes may be either ICB-induced rapid tumor growth (hyperprogression) or a rapid influx of immune cells followed by decreasing tumor size (pseudoprogression). There is great need to understand the biologies contributing to these phenomena so that we can better treat patients with ICB.

In an effort to identify endogenous T cell-intrinsic targets that could functionally interact with anti-PD-1 therapy, we performed a forward genetic screen to discover T cell mutations whose frequencies of mutation were greater under the selective pressure of anti-PD-1 therapy. This approach identified a truncation mutant of AP2-associated kinase 1 (*Aak1*) that was more frequently mutated in intratumoral T cells from mice receiving anti-PD-1 therapy than untreated controls. Therefore, we hypothesize that there is a functional interaction between anti-PD-1 and Aak1. If supported by experimental evidence, Aak1 may be leveraged to understand mechanisms of response to ICB and inform novel combinatorial therapeutic development.

The immunocompetent preclinical cancer models A20 (B cell lymphoma), EL4 (T cell lymphoma), B16 (melanoma), and MC38 (colon carcinoma) were used to measure the effect of combining pharmacologic Aak1 inhibition (Aak1i) and anti-PD-1 treatment on tumor growth. Consistent with prior work, anti-PD-1 slowed tumor growth significantly in A20, EL4, and MC38 tumors. However, we observed that the addition of the Aak1i to anti-PD-1 treatment exacerbated tumor

growth in all four cancer models, with tumors growing as quickly as untreated controls. These data strongly demonstrate functional interaction between anti-PD-1 and Aak1i. Importantly, Aak1i treatment of A20, EL4, and B16 cells *in vitro* did not alter the rate of tumor cell proliferation, suggesting that the increased tumor growth rate observed *in vivo* may be the result of the interaction between Aak1i and anti-PD-1 treatment. This provocative and highly novel finding also suggests that Aak1 could be of great interest in understanding the clinical phenomenon of hyperprogression.

In a pilot study designed to profile the tumor microenvironment using the A20 tumor model, we observed that Aak1i monotherapy significantly increased the ratio of intratumoral CD8+ to CD4+ T cells. Furthermore, single-agent Aak1i increased the ratio of CD27+ to CD27- T cells, suggesting that infiltrating T cells may be proliferating and differentiating more productively than in control mice. However, in mice treated with combination Aak1i and anti-PD-1, the ratio of CD27+ to CD27- T cells was instead reduced. Additional studies are required to elucidate the mechanism by which Aak1i diminishes anti-PD-1 efficacy, and perhaps contributes to hyperprogression.

Abrogating regulatory T cells overcomes tumor-specific T cell exhaustion and prevents metastatic pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDA) is the 4th leading cause of cancer related deaths and has a dismal 5-year survival rate of 10%. PDA lethality is attributed to late diagnosis, early metastasis, and therapeutic resistance. Metastasis can occur before the development of histologically detectable tumors and is a leading cause of cancer-related deaths. We identified

that pancreatic tumor cells derived from mice that resist immunotherapy (e.g., tumor escape variants, TEV) rapidly metastasize upon re-implantation into the pancreas of syngeneic and immunocompetent mice, reflecting the pathogenesis of human PDA. We show that TEVs retain the targeted tumor antigen, and despite a defect in IFNg-inducible MHC class I upregulation, TEVs remain sensitive to tumor antigen specific T cell-mediated lysis in vitro, suggesting TEVs may confer unique qualities in vivo to resist T cell killing. Using a peptide:MHC tetramer to identify the tumor-specific CD8 T cells, we identified that intratumoral T cells in TEV tumors have increased Granzyme B production and a reduction in prototypical exhaustion markers Pd1, Lag3, and Tox. Overexpression of Tap1 in TEV cells to restore normal MHC class I upregulation causes prototypical exhaustion of tumor-specific CD8 T cells. Notably, primary tumors from TEVs were significantly enriched for Foxp3+ Tregs as compared to parental tumors. Using a genetic model, we identified that Treg depletion resulted in a drastic reduction in tumor burden and metastasis and improved tumor-specific T cell function in TEV tumors. We are currently working to identify a population of anti-metastatic CD8 T cells that expand in the absence of Tregs. Investigation of tumor-cell intrinsic changes identified elevated expression of the receptor tyrosine kinase Ddr2 in multiple EVs compared to immunotherapy naïve cells. Ddr2 has been demonstrated to drive metastasis in other cancer models and is correlated with reduced patient survival in PDA and similar malignancies. As such, studies to knock out Ddr2 in TEVs or over express Ddr2 in immunotherapy naïve cells are underway and will test the hypothesis that Ddr2 drives metastasis in PDA. Preliminary studies show that Ddr2 overexpression in cancer cells leads to a higher frequency of intratumoral Tregs. In summary, Tregs are key drivers of both T cell exhaustion and immunosuppression in pancreatic cancer and may prove a valuable clinical target for tumors that evade immune checkpoint blockade. Moreover, studies are now underway to investigate tumor cell intrinsic drivers of metastasis in PDA, such as Ddr2, and their effects on the T cell anti-tumor response.

Adenosine-mediated Immune Suppression of T-cell proliferation in vitro requires Equilibrative Nucleoside Transporters and is rescued by CD73 inhibitors

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Adenosine (ADO) is a key immunosuppressive metabolite within the tumor microenvironment (TME). Adenosine triphosphate (ATP) released from dying cells is successively converted by

ectonucleotidases into adenosine monophosphate (AMP) by CD39 and ADO by CD73. Binding of ADO to the adenosine 2a and 2b receptors (A2a/bR), expressed by immune cells (T cells, NK cells, macrophages and dendritic cells) is believed to mediate immune suppressive effects; although a recent study pointed to a role for the Equilibrative Nucleoside Transporters (ENT) in blocking T cell proliferation (Festag et al. 2020). ENTs transport ADO inside cells where it can be successively converted into AMP, ADP, and ATP by adenosine kinase (AK) and reduced into dATP, which has been proposed to block proliferation. Given the interest in blocking immunosuppressive effects of ADO, we seek to understand the mechanism by which ADO decreases T cell proliferation *in vitro* and *in vivo*.

Using primary human CD4 or CD8 T cells stimulated with CD3/CD28, we measured T cell function (T cell proliferation and IFN-g production) in presence of AMP and a broad range of ADO pathway modulators, including CD73, A2a/bR, ENT and AK inhibitors. We found that the CD73 inhibitor AB680 rescued CD8 T cell proliferation and pro-inflammatory IFN-g production. As previously reported, antagonism of A2a/bR by AB928 only rescued IFN-g production *in vitro*. Interestingly, two ENT inhibitors (dipyridamole and NMBPR) and two Adenosine kinase inhibitors (ABT-702 and 5-iodotubericidin) rescued T cell proliferation in the presence of AMP. These observations confirm previously published data (Festag et al. 2020) and indicate that transport of ADO into the cell followed by its conversion to AMP is involved in the anti-proliferative effect of ADO *in vitro*. As predicted from these observations, NECA, an analog of ADO which cannot be converted into AMP, was unable to block T cell proliferation despite blocking IFN-g production. To investigate whether *in vivo* tumoral ADO also affects proliferation of tumor-infiltrating lymphocytes (TILs), we monitored TIL proliferation in the E.G7-Ova/OT1 syngeneic tumor model by BrdU incorporation. We found that proliferation of *ex vivo* activated OT-1 CD8 T cells adoptively transferred into syngeneic tumor-bearing mice were suppressed in the TME and that AB680 significantly rescued OT1 CD8 T cell proliferation. Proliferation of the host CD8 T cells present in the tumor also increased in presence of AB680.

In conclusion, by blocking the conversion of AMP to ADO, CD73 inhibitors are uniquely positioned in the ADO pathway to rescue both TIL proliferation and cytokine production. Further *in vivo* studies will be needed to directly link the role of ENT and AK to ADO-mediated block of TIL proliferation.

An extended CRISPR-Cas9 Knock Out Screen to Dissect Mechanisms of T Cell Exhaustion in Human Tumors

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Cancer immunotherapies, in particular immune checkpoint inhibitors and adoptive T cell transfer, have demonstrated remarkable anti-tumor efficacy. However, only a minority of patients responds to existing therapies. This resistance is in part attributed to the dysfunction of intra-tumoral T cells, also termed T cell exhaustion. Checkpoint inhibition only transiently elevates T cell effector function due to the lack of durable epigenetic reprogramming. Many features of and pathways to exhaustion remain controversial, in particular in the context of human cancer. Here we aim to identify genes involved in cancer-induced T cell exhaustion, which could be targeted to improve current immunotherapies.

To identify such genes, we have conducted a pooled CRISPR/Cas9 knock out screen in an ex vivo T cell exhaustion model (Trefny et al., Nat Comm, in press). Repetitively stimulated T cells in this model express a specific T cell receptor recognizing a cancer antigen and resemble exhausted T cells on a phenotypic and transcriptomic level. We first profiled the proteome of ex vivo generated exhausted T cells with MS proteomics to compile a library of 1'637 genes to be screened. Cells were co-transduced with a gRNA library, electroporated with Cas9 protein and subjected to repetitive stimulation. Functionally rescued IFNg+ CD107a+ and exhausted IFNg– CD107a– cells were sorted and submitted for gRNA sequencing.

We have identified multiple hit genes associated with rescued T cell function. We have performed single gene knock outs of selected hits in our exhaustion model and have confirmed improved cytokine secretion, expansion and cancer cell killing by edited T cells. We are currently conducting in vivo studies by targeting hit genes in T cells adoptively transferred to tumor-bearing mice. For one hit gene, we have already demonstrated decelerated tumor growth and prolongated survival when knocked out in ovalbumin (Ova)-specific OT1 T cells transferred to MC38-Ova bearing mice.

Prospectively, we aim to characterize the tumor immune environment of mice receiving edited T cells and to sample the transcriptome and epigenome of knock out cells to elucidate the mechanism by which these genes of interest contribute to T cell exhaustion and interact with other immune cells. Details of the genetic screen and mechanistic characterizations are provided at the meeting.

Analyzing susceptibility to macrophage activating immunotherapy by using patient-derived tumor explants and ovarian ascites cells

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As foremost regulators of cancer-related inflammation and immunotherapeutic resistance, tumor-associated macrophages (TAM) have garnered major interest as immunotherapeutic drug targets. A novel humanized antibody bexmarilimab, targeting the scavenger receptor Clever-1 on TAMs, has shown clinical benefit in ~30% of patients with advanced solid tumors (MATINS; NCT03733990). To identify components of the tumor microenvironment driving response to bexmarilimab we first characterized the phenotype and location of Clever-1 expressing macrophages in breast tumors by single-cell RNA sequencing and spatial profiling. Thereafter, patient-derived breast tumor explant cultures were treated with bexmarilimab and immune activation was measured with RNA sequencing and cytokine profiling. Our results show that Clever-1 mRNA (*STAB1*) expression was mostly identified on immunosuppressive *IL4I1*+ and *TREM2*+ monocyte-derived TAMs located in both lymphocyte rich and excluded areas in the tumor stroma. Bexmarilimab treatment induced TNFα and CXCL10 secretion in 30-40% of the treated explants. Characterization of the molecular features supporting bexmarilimab response showed that chronic interferon priming prevented Clever-1 blockade-induced macrophage activation. This effect was validated in ovarian ascites TAMs where high interferon signatures had a negative effect on bexmarilimab activity. These data suggest that bexmarilimab therapy has potential anti-tumor efficacy in tumors with a low pre-existing interferon response.

Anti-LAIR1 Antagonistic Antibodies Block Collagen-mediated Suppression of T Cell Activation

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Checkpoint inhibitors, especially anti-PD-1 drugs, have changed the cancer treatment landscape over the last decade, but most patients still do not respond to therapy. Myriad other cancer immunotherapies are currently under investigation to address additional aspects of the immunosuppressive tumor microenvironment, as well as addressing anti-PD-1 therapy resistance mechanisms to increase patient response to anti-PD-1 molecules.

Tumor associated macrophages (TAMs), and collagen promote an immune-suppressive microenvironment in solid tumors. Leukocyte Associated Immunoglobulin-like Receptor 1 (LAIR1) is an immune inhibitory transmembrane glycoprotein expressed on lymphocytes and myeloid cells. Known ligands for LAIR1 are collagen and proteins containing collagen-like domains, such as complement component 1q (C1q), and stromal protein Colec12. Here, we used a novel LAIR1 antagonist antibody designed to mobilize anti-tumor immunity by disrupting the tolerogenic LAIR1 pathway in collagen-rich solid tumors.

We demonstrated that immune checkpoints are upregulated in response to tumor stimuli. Immunosuppressive TAM-like M2 macrophages incubated with ascites from ovarian cancer patients upregulated expression of PD-L1 and LAIR1. Non-activated M0 macrophages that were differentiated into M2 macrophages with M-CSF and IL-4 in the presence of collagen and an isotype control antibody showed a large increase in PD-L1 expression. Similarly differentiated macrophages in the presence of a LAIR1 antibody exhibited significantly attenuated PD-L1 expression.

In an autologous monocyte derived M2 macrophage/T cell co-culture mixed lymphocyte reaction (MLR) in the presence of collagen, cells incubated with a LAIR1 antibody showed increased interferon gamma (IFNγ) production and T cell proliferation compared to cells incubated with an isotype control. In allogeneic MLRs on collagen-coated plates, cells were treated with an anti-LAIR1 antibody, an anti-PD-1 antibody, a combination of both antibodies, or isotype controls. Anti-LAIR1/anti-PD-1 combination treatment enhanced the secretion of the proinflammatory molecules GM-CSF, IFNγ, and TNFα significantly more than either monotherapy. Enhanced IL-6 secretion is a known resistance mechanism to anti-PD-1 therapy. In the allogeneic MLR, anti-LAIR1 therapy was able to significantly decrease the anti-PD-1 antibody-induced increase in IL-6.

In vivo, in an MDA-MB-231 tumor model in NSG-SGM3 mice humanized with CD34+ stem cells, anti-LAIR1 therapy significantly increased animal survival. Anti-LAIR1 treatment also significantly decreased the number of metastatic liver nodules compared to control-treated mice.

These data suggest that LAIR1 pathway blockade may reverse the tolerogenic effects of collagen on immune cells in the solid tumor microenvironment, demonstrating the therapeutic potential of anti-LAIR1 antagonistic antibodies as a monotherapy or in combination with anti-PD-1 therapies to overcome the IL-6 mechanism of resistance.

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B Cell Leukemia Elicits a Suppressive Tr1-like CD4 T cell Response that can be Reversed with Anti-PDL1-Based Combination Therapy

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CD4 T cell exhaustion has been associated with relapse in B cell leukemia (B-ALL) patients. However, checkpoint blockade therapy by itself has not been effective in treating B-ALL. Whether dysfunctional states other than exhaustion exist in leukemia induced CD4 T cells is still unknown. Using a murine model of B-ALL, we demonstrate that leukemia-induced exhausted CD4 T cells had a unique regulatory-cytotoxic phenotype (T_{rex}) with preferential expression of IL10 and c-Maf, which are markers of immunosuppressive Tr1 cells. Leukemia cells acting as APCs elicited CD4 T cells with a similar Tr1 phenotype that suppressed bystander T cells, suggesting that leukemia elicits Tr1-like CD4 T cells that actively promote an immune suppressive environment. While anti-PDL1 or nilotinib (targeted therapy) treatment alone had a minimal impact on survival, concurrent treatment with anti-PDL1 and nilotinib had a significant synergistic effect that was dependent on CD4 and CD8 T cells. Nilotinib treatment increased markers of helper function, but also regulatory function, in the T_{rex} cells. In contrast, treatment with nilotinib and anti-PDL1 induced expansion of a leukemia-specific CD4 T cell clone with lower expression of Tr1 markers (IL10, c-Maf) and higher expression of helper molecules that facilitate recruitment and activation of anti-tumor immune cells (CD40L, CCL4, TNF). This expanded CD4 clone had a helper-cytotoxic (T_{hctx}) phenotype and was associated with a greater proportion of $GzmB^+$ CD8 T cells in nilotinib + anti-PDL1 treated mice. Thus, combining nilotinib and anti-PDL1 alters the balance between immunosuppressive Trex and immune-activating T_{hctx}, thereby inducing a protective anti-leukemia immune response that prevents relapse.

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BALLkine-2: Localized delivery of cytokine IL-2 using porous nanoparticle drug delivery system, 'DegradaBALL', achieves durable tumor control with less systemic adverse effects in cancer immunotherapy

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Recombinant human interleukin-2 (rIL-2) has been considered as a highly potent cytokine in activating T cells and NK cells for cancer immunotherapy. Recent studies are focusing on the development of modifying the IL-2 receptor alpha (IL-2Ra) domain to reduce regulatory T cell (Treg) development. However, the most hostile limitation of the clinical utility using high-dose intravenous rIL-2 therapy is severe systemic toxicity such as vascular leak syndrome (VLS), pulmonary edema, and hypotension.

In this study, we develop peritumorally injectable 'BALLkine-2', which is rIL-2 loaded inside the pores of porous nanoparticle (DegradaBALL), to reduce systemic side effects of rIL-2 for immunotherapy by minimizing systemic IL-2 exposure. Notably, pharmacokinetics performed in cynomolgus monkeys revealed that subcutaneous (SC)-injection of BALLkine-2 not only dramatically reduces the Cmax and AUC of IL-2 in the blood, but also increases the half-life of IL-2 compared to IV- or SC-injection of free rIL-2. Interestingly, peritumoral (PT)-injection of BALLkine-2 maintained high concentration level of IL-2 (>100 ng/g) in the tumor up to 2 days, and IL-2 was still detectable with ~ 1 ng/g concentration up to 5 days after treatment in B16F10 melanoma xenograft tumor. Moreover, PT-injection of BALLkine-2 notably reduced lung edema and VLS in the B16F10 melanoma model. Importantly, the BALLkine-2 enhanced the recruitment of CD8+ T cells and activation of NK cells, but did not induce Treg cell development within the B16F10 tumor in vivo. BALLkine-2 more synergized with PD-1 blockade than high-dose rIL-2 administration in orthotopic and metastatic B16F10 melanoma model although smaller dose (4 vs 10 mg/kg) and fewer injections (4 vs 20 injections) are applied compared to systemic rIL-2 injection. Most interestingly, systemic administration of rIL-2 evoked the expression of PD-1, TIM-3, and LAG-3 on CD8+CD3+ T cells and its ligand, but PT-injected BALLkine-2 did not. Importantly, SC-injected BALLkine-2 was also observed in secondary lymphoid organs of the B16F10 melanoma model, such as spleen and draining lymph nodes, and increased the population of effector cells in secondary lymphoid organs, which leads to effective systemic anti-tumor immune response.

The advantages of BALLkine-2 over conventional high dose IL-2 therapy include i) convenient local injection imposing both local and systemic anti-cancer immune responses by higher exposure to the tumor and its distribution to the spleen and draining lymph nodes, ii) no notable development of Treg cells and immune exhaustion, and iii) lower total dosage (4 vs 10 mg/kg) and fewer injection (4 vs 20 injections) to elicit satisfactory therapeutic outcome, which can safely and efficiently strengthen synergistic effect in the combination therapy with aPD-1 Ab. We expect that BALLkine-2 will be a highly potent therapeutic option in cancer immunotherapy due to higher therapeutic efficacy with smaller and fewer doses, and remarkably reduced systemic toxicity compared to conventional high dose rIL-2 treatment.

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Batf3 dendritic cells and 4-1BB/4-1BB ligand axis are required at the effector phase within the tumor microenvironment for PD-1/PD-L1 blockade efficacy

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Anti-PD-1/PD-L1 antibodies reinvigorate T cells within the tumor microenvironment, which contributes to clinical efficacy as an immunotherapeutic. However, which positive signals are required for the T cell reinvigoration upon PD-1 blockade are incompletely understood. Using chimeric mice, we found that Batf3$^+$ dendritic cells (DCs) were required within the tumor microenvironment (TME) at the moment of anti-PD-L1 treatment for therapeutic efficacy. Flow cytometric analysis, gene-targeted mice, and blocking antibody studies revealed that 4-1BBL was a major positive costimulatory signal provided by these DCs that mediated anti-PD-L1-induced tumor growth control, antigen (Ag)-specific CD8$^+$ T cell expansion, and functional reinvigoration. In human tumor samples, Batf3$^+$ DCs and CD8$^+$ T cells were found to co-localize, which correlated with anti-PD-1 efficacy. Furthermore, spatial transcriptomics on human tumor samples showed that CD8$^+$ T cells in close proximity with Batf3$^+$ cells expressed transcripts indicating expression of multiple inhibitory receptors, a stem-like phenotype, and effector-like states that have been shown to correlate with anti-PD-1 efficacy. In contrast, CD8$^+$ T cells distant from Batf3$^+$ cells lacked these markers. Our results demonstrate that Batf3$^+$ DCs are crucial for the reinvigoration of T cells upon PD-1 blockade within tumor sites, and indicate a critical role for the 4-1BB/4-1BBL axis within the TME during this process.

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Bhlhe40 Regulates T Cell Effector Function to Orchestrate Tumor Microenvironment Remodeling and Efficacy of Immune Checkpoint Therapy

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Despite the notable success of immune checkpoint therapy (ICT) in curing certain patients with various types of cancer, continued research is needed in order to find novel molecular targets and biomarkers in order to improve the efficacy of immunotherapy for more patients. We previously found the transcription factor Bhlhe40 is upregulated in tumor antigen-specific CD8+ and CD4+ T cells within the tumor microenvironment in response to ICT. Both global (Bhlhe40-/-) and T cell-specific (CD4-Cre+ Bhlhe40f/f) genetic deletion of Bhlhe40 in 1956 sarcoma- or B16-OVA melanoma-bearing mice resulted in failure to respond to anti-PD-1 or anti-CTLA-4 ICT. At the same time, Bhlhe40 deletion in macrophages and granulocytes using LysM-Cre+ Bhlhe40f/f mice did not affect ICT-driven tumor rejection. Furthermore, using a low-dose primary 1956 tumor injection, combination of anti-PD-1 and anti-CTLA-4 ICT, and a secondary 1956 tumor rechallenge (50 days after primary tumor rejection) protocol, we found that rejection of a secondary 1956 tumor rechallenge was dependent upon Bhlhe40, indicating defective anti-tumor immune memory in the absence of Bhlhe40. We next profiled intratumoral immune cells from 1956 tumor-bearing Bhlhe40+/+ and Bhlhe40-/- mice treated with control, anti-PD-1, or anti-CTLA-4 mAb using multiple approaches, including scRNA-seq, and found ICT-induced tumor microenvironment remodeling of both myeloid and lymphoid cell populations in response to ICT was dependent upon Bhlhe40. While loss of Bhlhe40 had only minor effects on the relative proportion of intratumoral CD4+ and CD8+ T cells under all treatment conditions, these populations were characterized by transcriptional, proteomic, and functional changes within corresponding subpopulations in the absence of Bhlhe40. Changes associated with the loss of Bhlhe40 occurred in multiple clusters of CD4+ and CD8+ T effector cells and included alterations in expression of transcripts encoding granzymes, chemokines/chemokine receptors, and immunoregulatory proteins (Tigit, Lag3), as well as dysregulated NF-kB signaling, actin cytoskeleton remodeling, and glycolysis and glucose transport. Consistent with transcriptomic data, intratumoral Bhlhe40-/- CD4+ and CD8+ T cells from mice treated with ICT display a significant decrease in extracellular acidification rate (ECAR), indicative of reduced aerobic glycolysis in the absence of Bhlhe40. Notably, both CD4+ and CD8+ effector T cells lacking Bhlhe40 displayed defective ICT-induced IFN-g production. Chromatin immunoprecipitation (ChIP) using isolated intratumoral CD4+ and CD8+ T cells from mice undergoing ICT indicated Bhlhe40 directly bound known Ifng promotor elements, suggesting that direct regulation of Ifng transcription by Bhlhe40 may be occurring in intratumoral T cells. The alterations observed within T cell subpopulations in the absence of Bhlhe40 were also associated with profound defects in ICT-induced macrophage remodeling from a CX3CR1+ CD206+ subpopulation to an iNOS+ subpopulation that is typically observed during effective ICT in this tumor model. Collectively, our data demonstrate the role of Bhlhe40 in the ICT-induced antitumor immune response, pinpoint potential pathways and effector mechanisms under its control, and the potential of Bhlhe40 as a biomarker, and a target for immunotherapy.

Bile acids acts as a metabolic checkpoint within liver tumor that imparts resistance to immunotherapy

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The success and failure of immune-checkpoint blockade depends heavily on generation of effector CD8+ T cell responses and their recognition of target tumor cells. Despite recent developments in the identification of novel immune checkpoints, majority of the tumors and/or patients fail to respond to or only partial respond to immune checkpoint blockade. Hepatocellular carcinoma is one such tumor despite being infiltrated by CD8+ T cells, fail to respond to immune checkpoint blockade as well as adoptive T cell transfer therapies. Often liver cancers are presented with elevated levels of bile acids (BAs) which can directly or indirectly contribute to tumor progression. However, the contribution of these BAs in blunting tumor-specific T cell responses even in the presence of immune checkpoint blockade has been poorly investigated. *We have identified that the metabolic state of liver cancer cells enables them to evade immune recognition by regulating bile acids levels within tumor microenvironment.* Using various mouse models of liver cancer and human hepatocellular carcinoma patients, we observed that tumors displayed increased levels of conjugated BAs as well as microbial derived secondary BAs. Interestingly, these BAs are specifically enriched within tumor regions where tumor-specific T cells are abundant. To determine the role of tumor driven BAs synthesis on anti-tumor immunity, we have knocked out every step of the BA synthesis pathway using AAV-mediated Crispr-Cas9 system and observed that the rate-limiting enzyme of BA synthesis pathway - CYP7A1 and BA conjugating enzyme BAAT are essential for regulating BA levels and T cell responses within tumors. Thus, tumor-specific knockdown of CYP7A1 or BAAT resulted in significant reduction in conjugated BAs and altered levels of secondary BAs which is associated with increased tumor-specific T cell responses (both survival and function). More importantly, combining BAAT knockdown with anti-PD-1 resulted in complete elimination of tumors, whereas anti-PD-1 alone did not influence T cell responses or tumor burden. The therapeutic benefit of modulating BA synthesis pathway was dependent on the direct effects of BAs on T cells as tumor specific-T cells within liver accumulated BAs. Additionally, culturing in vitro activated CD8+ T cells with various BAs indicated that while primary conjugated BA (mainly TCDCA) dose-dependently affected T cell survival, secondary BA (especially LCA) inhibited T cell effector functions. Intriguingly, TCDCA induced mitochondrial oxidative stress in T cells leading to inhibition mitochondrial respiration, and Reactive Oxygen Species (ROS) induced cell death. Hence, supplementing CD8+ T cells with N-Acetyl Cysteine (antioxidant) or over-expression of catalase inhibited TCDCA induced T cell death. Subsequently, adoptive transfer of Catalase over-expressing T cells improved their fitness within liver tumors. On the other hand, LCA induced ER

stress and calcium driven NFAT-Nur77 signaling leading to T cell dysfunction. Consequently, tumor-specific T cells lacking Nur77 lead to improved effector functions within liver tumors. Taken together, we have identified a bile acid synthesis pathway as a novel metabolic checkpoint mechanism by which liver tumors derived BAs impede tumor-specific T cell responses and thwart the efficacy of immune checkpoint blockade by directly inhibiting T cell survival and function.

Blocking the CD47 'don't eat me' signal in tumors with T cells engineered to secrete SiRPα decoys

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To extend the efficacy of adoptive T cell transfer (ACT) against a broader range of cancers and for a large proportion of patients it is widely held that endogenous immunity must be harnessed. The transmembrane protein CD47, oftentimes referred to as the 'don't eat me' signal, is ubiquitously expressed by healthy human cells and serves to inhibit their phagocytosis by myeloid cells via engagement of the inhibitory receptor Sirpa. CD47 is also frequently upregulated by tumors and the CD47/Sirpa axis is now recognized as a major immune checkpoint of the myeloid compartment. Although systemic blockade of this axis can confer important anti-tumor effects by 'releasing the brakes' on macrophages, it is highly also toxic. To overcome this obstacle, we coengineered tumor-redirected TCR-T cells to secrete a high-affinity Sirpa -Fc decoy directly (under 6xNFAT) in tumors. Notably, we employed an affinity-optimized HLA-A2 /NY-ESO-1 specific TCR (A97L) which itself conferred superior effector function, as well as engraftment and tumor control, by engineered T cells as compared to the wild-type TCR-T cells. Co-engineering of the A97L TCR-T cells with a high-affinity Sirpa-Fc decoy enabled better control of tumor outgrowth in a Winn assay, but for subcutaneous tumor models in NSG mice we observed macrophage-mediated depletion of the T cells which themselves express CD47.

However, we found that T cells gene-modified to secrete a Sirpa monomer decoy were not depleted by human macrophages *in vitro*. Moreover, we demonstrated that the combination of tumor-specific monoclonal antibodies bearing a functional Fc tail (i.e. providing a 'come eat me' signal), including Cetuximab and Avelumab, synergized with Sirpa monomer secreted by TCR-T cells to augment phagocytosis of target tumor cells. Notably, we also observed significantly improved solid tumor control by A97L TCR-T cells upon coadministration with Cetuximab or/and Avelumab. Taken together, our data indicate that co-administration of Cetuximab or/and Avelumab along with T cells gene-modified to express an affinity optimized TCR and to secrete Sirpa decoys is a promising strategy to improve clinical outcome to ACT.

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Cancer-derived secretory succinate as a theranostic target in cancer

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The importance of metabolism in cancer has become an emerging theme in cancer research. Within the tumor microenvironment, cancer cells release soluble molecules not only to initiate oncogenic signaling for their development but also to impact the surrounding cells, including the immune cells, to enhance tumor progression. By using a comparative metabolomics analysis coupled with molecular cellular biochemical approaches and animal models to identify and determine succinate as a novel factor secreted by cancer cells which triggers TAM polarization and promotes cancer metastasis *in vitro* and *in vivo*. Importantly, compared with healthy subjects, serum succinate levels were elevated in patients with non-small cell lung cancer (NSCLC). Accordingly, we hypothesize that depletion or neutralizing of serum succinate by anti-succinate antibodies suppress tumorigenesis. We performed antibody engineering methods to successfully generate mouse succinate monoclonal and humanized antibodies which can specifically recognize and neutralize succinate and suppress TAM polarization and tumorigenesis including migration and metastasis. Thus, these succinate antibodies may sever as a theranostic antibodies for cancer therapy.

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CD19 CAR-T Therapy in Solid Organ Transplant Recipients: Illustrative Case and Systematic Review

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Post-transplant lymphoproliferative disorder (PTLD) is a leading cause of cancer death in solid organ transplant recipients (SOTRs). Despite improved management strategies for newly diagnosed PTLD, relapsed or refractory (R/R) PTLD portends a high risk of death and effective management is not well established. CD19-targeted CAR-T cell therapy has been utilized in the treatment of aggressive B-cell malignancies, but the risks and benefits are unknown for the management of PTLD occurring in SOTRs. We present an illustrative case and systematic review of SOTRs with PTLD treated with CD19 CAR-T therapy. A case of diffuse large B-cell lymphoma (DLBCL) PTLD was treated at our institution with lisocabtagene maraleucel. This patient achieved a complete response (CR) with limited toxicity but experienced a $CD19^+$ relapse 8 months after infusion despite CAR-T persistence. Literature review revealed 14 DLBCL and 2 Burkitt lymphoma PTLD cases treated with CD19 CAR-T cells. Kidney (n=12), liver (n=2), heart (n=2), and pancreas after kidney (n=1) transplant recipients were analyzed. The median patient age was 46 years with 12 males (70.6%) and 5 females (29.4%). Most cases were Ann Arbor stage 4 (13/17, 76.5%) and EBV-negative (14/17, 82.4%). CAR-T products used included axicabtagene ciloleucel (n=11), tisagenlecleucel (n=3), lisocabtagene maraleucel l (n=1), and 2 study products (ChiCTR2000032211 and ChiCTR1800019622). The objective response rate (ORR) was 82.4% (14/17), with 58.5% (10/17) CRs and a 6.5-month median duration of response. Among kidney transplant recipients, the ORR was 91.7% (11/12). Allograft rejection occurred in 4 patients (23.5%), all of whom were kidney transplant recipients. Most patients developed CRS (15/17, 88.2%), though most cases were grade 1 (11/15, 73.3%). Grade ≥3 ICANS occurred in 29.4% (5/17) and 36.4% (4/11). No cases of graft failure or rejection-/treatment-related mortality occurred. Our analysis suggests that CD19 CAR-T therapy offers short-term effectiveness and manageable toxicity in SOTRs with R/R PTLD. Further investigation through larger datasets and prospective study is needed.

CD3-Siglec15 BITE therapy inhibit tumor growth and increases T cell infiltration in human pancreatic cancer

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Bispecific T cell engager (BiTE) antibody constructs, designed to redirect T cells to induce lysis of tumor cells through simultaneous binding to CD3 on T cells and to a tumor associated antigen, has shown considerable promise in B cell malignancies. Unfortunately, BiTE has generally been less effective for solid tumors such as pancreatic ductal adenocarcinoma (PDA), a devastating malignant disease with a dismal prognosis characterized by both a dense stroma and an immunosuppressive tumor microenvironment (TME). Here, we engineered a Siglec-15 (S15) targeted BiTE and evaluated its effectiveness in vitro and in vivo. S15 is upregulated in various human cancer cells (including PDA) and tumor-infiltrating myeloid cells, and its expression appears mutually exclusive to PDL-1. S15 enhances the immunosuppressive TME by suppressing macrophage-associated T-cells in tumors. Elevated expression of S15 correlates with low survival in PDA patients. In NSG mice tumor bearing mice, S15/CD3 BiTE effectively limited tumor growth, in marked contrast to tumors in mice receiving either anti-S15 or anti-CD3 mAbs. This is likely due to breaking down the stromal barrier: S15 is also over-expressed on stroma cells surrounding PDAC, and S15 targeted biTE greatly reduced the stromal barrier in PDAC leading to much greater T-cell infiltration. We also observed a less immunosuppressive TME as well as a substantial increase in T-cells in the circulation. Taken together, these results underscore S15/CD3 BiTE as a promising immunotherapy for PDA patients in the future.

CD59 upregulation by NANOG promotes resistance to complement dependent cytotoxicity on cancer cells

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Cancer immunoediting triggered the adaptation of tumor cells to the host's immune system, thereby contributing to the generation of cancer cells with better survival advantages. Previously, we demonstrated that cytotoxic T lymphocyte (CTL)-mediated immune pressure enriches NANOG+ tumor cells with immune-refractory properties that make them resistant to CTLs. Here, we found that CTL-mediated immune pressure triggers cross-resistance of tumor cells to complement system, a part of the innate immune system. Mechanistically, NANOG upregulates membrane complement regulatory proteins (mCRPs), including CD59, through transcriptional induction, thereby contributing to the resistance of tumor cells against complement-dependent cytotoxicity (CDC). Importantly, targeting of NANOG sensitized immune-refractory tumor cells to trastuzumab-mediated CDC. Thus, our findings implicate the

possible mechanism by which selection imposed by T-cell based immunotherapy triggers complement-resistant phenotypes in the tumor microenvironment, by establishing a firm molecular link between NANOG and CD59 in the immune-edited tumor cells.

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CD8 T cell intrinsic Helios restrains tumor-specific T cells following immune checkpoint blockade in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) is a lethal malignancy with a dismal 5-year survival rate that is resistant to immune checkpoint blockade. Here, we utilize an orthotopic KPC tumor model that expresses a defined model neoantigen to track the fate of tumor-specific T cells using a peptide:MHC tetramer during anti-PD-L1 therapy. We identify that immune checkpoint blockade elicits a unique tumor-specific CD8 T cell cluster in the spleen that expresses Helios (*Ikzf2*) by scRNAseq. We validate and further characterize the splenic Helios+ tumor specific T cells that are elicited by anti-PD-L1 by flow cytometry. In the spleen, tumor-specific Helios+ T cells are CD44hi and Klrg1- and in the tumor, Helios+ antitumor T cells co express exhaustion markers PD1 and Lag-3. We show that Vav-Cre x *Ikzf2*$^{fl/fl}$ mice in which Helios is absent from all immune cells have significantly blunted orthotopic tumor growth and prolonged survival following anti-PD-L1 therapy. In addition, a higher proportion of tumor-specific CD8 T cells express the effector marker Klrg1 in the tumor in Vav-Cre x *Ikzf2*$^{fl/fl}$ mice. By generating mixed bone marrow chimeric mice in which 50% of the bone marrow is from Vav-Cre x *Ikzf2*$^{fl/fl}$ mice and 50% of the bone is wild type, preliminary data support that anti-PD-L1 retains efficacy in this setting and *Ikzf2*$^{-/-}$ CD8 T cells express IFNg to a greater degree than wild type T cells following *ex vivo* antigen specific restimulation. Taken together, our results suggest that CD8 T cell intrinsic Helios restrains antitumor CD8 T cell effector function following immunotherapy in PDA and may be a target for developing effective immunotherapies for this lethal disease.

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Cellular Fragmentation as a Putative Mechanism for the Enhanced Immunogenicity of Irreversible Electroporation Cancer Lysates

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Focal cancer therapies such as irreversible electroporation (IRE), thermal ablation, and cryoablation are attracting attention for their potential to induce systemic immunity, acting as "*in-situ* vaccines". Interestingly, different modalities of focal therapy generate differential T-cell proliferation in vitro and overall protection in vivo, with irreversible electroporation often showing the strongest response. We hypothesized that this difference is linked to increased dendritic cell activation and uptake of cellular debris upon exposure to IRE lysates. As the interaction between the innate immune system and ablated cancer cells is poorly understood, the following study aimed to assess dendritic cell function after exposure to focally ablated B16-F10 cancer cells as well as characterize the biochemical and biophysical features of the lysates to understand potential mechanisms for enhanced dendritic cell performance.

Flow cytometry analysis of dendritic cells incubated overnight with B16-F10 lysates from different ablation methods indicated that dendritic cells phagocytosed more cellular debris from IRE lysates and were phenotypically more activated compared to dendritic cells incubated with cryoablation or thermal ablation lysates. Analysis of lysate supernatants with various biochemical assays showed that cryoablation caused greater cellular release of soluble immunostimulatory signals (ATP, HMGB1, TRP2 antigen) than IRE or thermal ablation. However, immunostaining of whole cell lysates suggested that larger immunomodulatory biomolecules including dsDNA and exposed F-actin were more prevalent in IRE lysates. From a biophysical perspective, light microscopy and nanoparticle tracking analysis revealed that IRE lysates contained a significantly higher amount of micron-sized cellular fragments and microparticles, respectively. Confocal microscopy confirmed that these cell fragments contained antigen and were efficiently taken up by dendritic cells. These findings suggest that while soluble signals are commonly assessed markers of immunogenicity, a more comprehensive analysis of lysate particle size and biophysical presentation of signals may be more predictive of downstream immune activation.

This study establishes that higher rates of T-cell proliferation engendered by IRE lysates as seen in literature are associated with greater dendritic cell activation and uptake of lysate debris. Further, the chief hallmark of IRE lysates appears to be extensive cellular fragmentation

resulting in increased dsDNA release as well as higher levels of microparticle-sized debris. Additional studies to interrogate a potential causative role of these two factors are in progress. Overall, this study provides valuable insight into how the modality of focal therapy affects the immunogenicity of the ablated cancer cells, with the potential to improve future ablation-based cancer vaccine designs.

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Characterization of determinants of effective adoptive cell therapy in B16F10 melanoma

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Adoptive cell transfer (ACT) of neoantigen-reactive CD8+ T cells has had some success in the clini is hampered by the immunosuppressive tumor microenvironment (TME) and limited understand what constitutes an ideal, targetable neoantigen. Murine models of anti-tumor CD8+ T cell immu the context of artificially introduced or overexpressed-self (tumor associated antigens, or TAAs) antigens are widely used, but this fails to recapitulate tumor-specific neoantigen-reactive CD8+ T that are crucial for tumor clearance. To address this gap, we developed a model of neoantigen-re CD8+ T cell immunity in the immunotherapy-resistant murine melanoma tumor, B16F10. We ider high-affinity T cells reactive to a neoantigen, Hsf2, as well as novel T cell receptor (TCRs) recognizi previously characterized TAAs such as gp100, Tyrp1, Trp2. The antigens and corresponding neoantigen- and TAA-reactive T cells studied comprise a full spectrum of attributes, ranging from to high antigen expression by the tumor and low to high T cell avidity, respectively. *In vitro*, we observed that killing of tumor cells by either neoantigen- or TAA-reactive T cells is dependent upo both tumor antigen expression and TCR avidity in tandem; tumor cell death was observed only w tumor antigen expression and TCR avidity were mutually high. Correspondingly, tumors highly expressing TAAs or neoantigens were targetable by ACT upon transfer of high-affinity T cells, yiel reduction in tumor growth relative to that observed in control mice. Further research is being conducted to understand the relative contributions of antigen abundance and TCR avidity as fact impacting ACT efficacy and T cell phenotype *in vivo*. We anticipate that this knowledge can be app towards optimization of antigen selection and ACT design in the future.

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Characterization of the immunogenicity of human neoantigens in vivo

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Neoantigens have been implicated as target during effective cancer immunotherapy, e.g. by immune checkpoint inhibitors or adoptive T cell therapy. Additionally, vaccine trials started where patients are treated with a multitude of predicted neoantigens. Neoantigens, mostly single non-synonymous point mutations, are often defined by a high predicted peptide-MHC (pMHC) affinity, yet only a low percentage of predicted neoantigens are in fact immunogenic, e.g. processed and presented. Relative to tumor mutational load, tumor-infiltrating lymphocytes against only few neoantigens are typically detected. Since somatic mutations creating a neoantigen occur sequentially over an extended period of time, the question arises, whether immunodominance obscures neoantigen-specific T cell responses. As neoantigens are difficult to define, we argue that immunogenicity in the strict sense can only be analyzed *in vivo*. Here, we analyzed the immunogenicity of naturally occurring patient-individual cancer neoantigens, which had elicited specific CD8 T cell responses in the respective patients. Simultaneous expression of 16 neoantigens in a spontaneous, low-immunogenic tumor in mice with a diverse human T cell receptor (TCR) repertoire restricted to HLA-A*02:01 resulted in cancer cell rejection or selection of antigen-negative variants and effective T cell activation was shown in all mice. The CD8 T cell response was directed against one dominant and occasionally low responses to 2-3 other neoantigens. TCRs specific for the dominant neoantigen used a restricted set of Vb/Va-Ja gene segments, contained almost no nucleotide insertions, had short CDR3 and shuffling Vb/Va chains between individual T cell clones retained specificity as long as the Vb CDR3 contained a certain germline-encoded D-segment motif. Thus, T cells against the dominant neoantigen occurred at a high precursor frequency in the naïve repertoire, which may explain the immunodominance. The dominant neoantigen prevented T cell responses against inferior neoantigens. Further analysis of neoantigen-specific TCRs is ongoing.

Chemotherapy enhances LAG-3 expressing progenitor exhausted (PD 1+SLAMF6+) CD8+ T cells which can be targeted by anti-LAG-3 and anti-PD-1 immune checkpoint blockade to improve anti-tumor immunity

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Immune checkpoint blockade (ICB) therapies that target CTLA-4, PD-1 or PD-L1 pathways have shown durable anti-tumor responses in a subset of cancer patients. However, as a stand-alone therapy, objective response rates only occur in a minority of patients, especially in aggressive cancers like mesothelioma. ICB that targets other immune checkpoints (e.g. LAG-3, TIM-3, TIGIT) have potential to improve the anti-tumor immune response and are currently being trialed across many cancer types. However, it still remains unclear if these will provide any further benefit when combined with chemotherapy.

Chemotherapy remains standard care for majority of aggressive cancers, and is widely used in combination with ICB. Many chemotherapeutics induce a variety of isolated immunostimulatory effects, including enhancing $CD8^+$ T cell proliferation and activation. The differentiation state of tumor infiltrating $CD8^+$ T cells is crucial for the success of ICB. Anti-PD-1 ICB acts on a subset of $CD8^+$ T cells that retain proliferative and cytotoxic capacity despite being suppressed within the tumor microenvironment. This subset of $CD8^+$ T cells exist in a progenitor exhausted (T_{PEX}) differentiation state and is normally characterized by the expression of surface PD-1 and SLAMF6 and, transcription factor TCF1. Despite chemotherapy being routinely administered in combination with ICB to cancer patients, it is unknown how chemotherapy changes the frequency and phenotype of T_{PEX} tumor infiltrating $CD8^+$ T cells. We hypothesized that chemotherapy induces phenotypic changes of $CD8^+$ T_{PEX} in the tumor microenvironment, with increased expression of inhibitory checkpoints which could be targeted with ICB to improve the anti-tumor immune response.

To investigate this, we performed multi-parameter flow cytometry to characterize the dynamics of tumor infiltrating $CD8^+$ T_{PEX} cells over multiple doses of 5-Fluoruracil (5-FU) or gemcitabine and in murine mesothelioma (AB1-HA) and colon cancer (CT26) models. 5-FU or gemcitabine alone delayed tumor growth but were non-curative. Chemotherapies increased tumor infiltrating $CD8^+$ T_{PEX} in both models, and increases in T_{PEX} were largely restricted to tumor antigen (HA) specific T cells. Both 5-FU and gemcitabine chemotherapies significantly increased expression of inhibitory checkpoint LAG-3 on $CD8^+$ T_{PEX} as 32.0 ± 5.5% and 63.7 ± 9.92% of $CD8^+$ T cells expressed LAG-3, PD-1 and SLAMF6 in 5-FU and gemcitabine treated tumors respectively compared to 5.62 ± 1.21% in PBS controls. There were no significant differences in expression of TIGIT, TIM-3, CTLA-4 or 41BB on $CD8^+$ T_{PEX} after chemotherapy. In addition, $LAG\text{-}3^+$ expressing

$CD8^+$ T_{PEX} retained proliferative capacity in chemotherapy treated tumors, with over 60% expressing Ki67 compared to 5% in PBS controls. To determine if LAG-3 expressing $CD8^+$ T_{PEX} could be targeted to improve tumor control, we administered anti-LAG-3 and anti-PD-1 ICB after two doses of 5-FU or gemcitabine and found both combination chemo-immunotherapies resulted in complete cures in 60% of mice compared to 0% in chemotherapy or ICB monotherapy controls. By studying the dynamic changes of $CD8^+$ T_{PEX} cells in two murine models, we identified ICB targets to combine with chemotherapy. Combination anti-LAG-3 and anti-PD-1 with chemotherapy could be a beneficial treatment option to combine with chemotherapy for aggressive cancers like mesothelioma.

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Cholesterol mobilization governs dendritic cell maturation and the immunogenic response to cancer

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As the chief antigen-presenting cells of the hematopoietic lineage, dendritic cells (DCs) residing in tissues are responsible for surveying the microenvironment, then educating T cells of acquired antigens. This process requires for either of the two primary subsets of conventional DCs, type I (cDC1) and type II (cDC2), to enter an activated, or mature, state involving the migration of DCs to lymphoid organs. Therefore, these migratory DCs have represented the canonical state of mature DCs. However, the precise subcellular processes underlying DC maturation have remained poorly understood. We previously showed that the gene program of migratory DCs, which we revised as the 'mature DCs enriched in immunoregulatory molecules' or mregDC state, is engaged upon phagocytosis of cell debris. As it reflects both immunostimulatory and regulatory potential, identifying the molecular pathways governing DC maturation into the mregDC state and the possible links that couple these dual features will aid in the design of future DC-targeted therapies that leverage the immunostimulatory activity of DCs without interference from their regulatory functions. We found that the mobilization of cholesterol, derived from the extracellular environment, via the intracellular cholesterol transporter NPC1 and their organization into lipid nanodomains on the DC cell surface mediates DC maturation. Inhibiting this process impairs the activation of T cells by mregDCs. Importantly, we identified) the receptor tyrosine kinase AXL as a central regulatory of the cholesterol-

dependent maturation into the mregDC state. The genetic deletion of AXL or its pharmacological inhibition with Bemcentinib amplified the repartitioning of cholesterol to the cell surface of mregDCs and increased the expression of CCR7, MHC molecules, and the IFN- receptor at the DC cell surface. Accordingly, in a model of primary lung adenocarcinoma, treatment with Bemcentinib or deletion of AXL from DCs generated a significantly more potent T cell-dependent anti-tumor response, demonstrating the therapeutic potential in targeting AXL to harness the immunostimulatory potential of mregDCs and boost anti-tumor immunity.

Combined PARP Inhibition and DNA Alkylation as a Mechanism to Enhance Gamma Delta T Cell Mediated Immunotherapy in Glioblastoma

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Glioblastoma (GBM) remains one of the deadliest malignancies partially due to the ineffective treatment modalities. Survival of GBM tumors amidst aggressive standard of care (surgical resection, chemo-, radio-therapy) relies on the acquired adaptive mechanisms of GBM cells to overcome both intrinsic and extrinsic stressors. However, if stressful stimulus remains unmitigated, immune recognition via upregulation of stress responsive NKG2D ligands (NKG2DL) promote immune cell trafficking of NKG2D receptor bearing immune cells such as natural killer cells and gamma-delta T cells. Leveraging the induction of NKG2DL to enhance immunotherapeutic approaches has shown extraordinary promise in a variety of cancers, including GBM. Specifically, the DNA alkylating agent, temozolomide (TMZ), was previously shown to induce NKG2DL expression and promote gamma-delta T cell mediated cytotoxicity. Expanding on these initial findings, we sought to enhance the stress inducing potential of TMZ by inhibiting compensatory DNA repair mechanisms that may minimize overall effectiveness. Therefore, we hypothesized that combined PARP inhibition (niraparib) and DNA alkylation (TMZ) in GBM would more greatly induce NKG2DL and increase gamma-delta T cell mediated cell death. Using cells isolated from glioblastoma patient derived xenografts (PDX) or a syngeneic mouse glioma model, we first confirmed that the combination of niraparib and TMZ decreased cell growth. To determine if there were additional immuno-modulatory benefits of niraparib and TMZ, we performed RT-PCR and evaluated transcriptional changes of either human or murine NKG2DL genes. Data suggested heterogeneous mRNA induction

of NKG2DL, although significant increases in ULBP1 (human) or MULT-1 (murine) were observed in two of the three cell types tested. To validate our gene expression findings, we confirmed via flow cytometry that there were protein level expression changes of ULBP1 and MULT-1. Furthermore, using SYTOX Orange Nucleic Acid Stain based flow cytometry we determined the combinatorial benefit of niraparib and TMZ to enhance gamma delta T cell mediated killing of PDX-derived cells following co-culture. Collectively, our data warrants utilization of combined DNA alkylation and PARP inhibition for NKG2D based immunotherapy in GBM. Interestingly, our findings do suggest cellular heterogeneity in responsiveness to NKG2DL inducing stimuli which may have mechanistic underpinnings worthy of further investigation.

Comprehensive immune landscape reshaped by anti-CTLA4 antibody treatment in a mouse tumor transplant model

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Anti-CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) immune checkpoint inhibitor is the first inhibitor (ICI) approved for the treatment of advanced melanoma and has since been applied to other types of cancer. The primary target of anti-CTLA-4 antibodies is CD8+ cytotoxic T lymphocytes (CTLs) and the treatment releases inhibitory function of CTLA4 leading to CTL activation and efficient killing of tumor cells. Other immune cell populations are likely to undergo functional alterations that may contribute to the activation of CTLs and provide additional mechanisms of immune activation. To depict a comprehensive immune cell landscape reshaped by the CTLA-4 checkpoint inhibitor, we performed single-cell RNA sequencing in a mouse syngeneic tumor transplant model. After CTLA-4 inhibition, massive immune cell infiltration accompanied tumor regression, with a dramatic increase in T and B/plasma cell proportions. We found that B cells in tumor transplant represented follicular, germinal center, and plasma B cells. Some of these B cells were found to have identical B cell receptor clonotypes and were reactive to the tumor. The regressed tumor contained tertiary lymphoid-like structure with intermingling T and B cells, suggesting a role of antigen presenting B cells in the T cell activation. Taken together, our data provide a panoramic view of the immune

microenvironment after CTLA-4 inhibition and suggest a role for tumor-specific B cells in antitumor immunity.

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Conditioning local lymph node environments with synthetic depots promotes tolerogenic antigen presenting cell function

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In Multiple Sclerosis (MS), the immune system degrades the protective matrix, myelin, on neurons in the central nervous system. Currently, many MS treatments broadly restrain immune function, limiting a patient's ability to fight infection. A new approach is to engineer antigen specific tolerance by promoting regulatory functions in immune cells. Before T cells commit to inflammatory or regulatory functions, antigen presenting cells (APCs) such as dendritic cells (DCs) and B cells provide signals to influence T cell development in the lymph node (LN). Our lab has synthesized microparticles (MPs) co-encapsulating a myelin antigen, MOG, and a regulatory molecule, rapamycin (rapa). These MPs are injected directly to LN but are designed to be too large to passively drain away following injection; instead, they degrade and release tolerizing cues to LNs. A single injection of MOG/rapa MPs at the peak of disease reversed paralysis in a mouse model of MS. This exciting finding led us to investigate how MPs alter DC and B cell function to support tolerance.

We hypothesized MPs deliver encapsulated antigen to APCs for presentation. To test this idea, DCs and B cells were isolated and treated with MPs encapsulating model antigens. DCs and B cells presented encapsulated SIINFEKL in MHCI and Eα in MHCII. Furthermore, co-encapsulating rapamycin inhibited antigen presentation and downregulated costimulatory molecules. These experiments demonstrated that MPs alter key T cell stimulation functions on B cells and DCs, thus we tested the impact that MP-conditioned APCs have on downstream T cell development. Antigen specific T cells were co-cultured with MP treated APCs before measuring T cell proliferation and phenotype. These studies showed that encapsulating antigen drove proliferation while coencapsulating rapamycin reduced proliferation and promoted T_{REG} phenotype among cells that did proliferate. We hypothesized that these cellular changes would be reflected by structural changes in the lymph node (LN). To examine how intra-LN (iLN) MP treatment alters LN structure, mice were treated with MPs containing no cargo or containing

MOG/rapa. These studies showed rapid restructuring of/ tolerance-associated laminin-α4 and inflammation-associated laminin-α5. Treatment with MOG/Rapa MPs increased laminin-α4 relative to laminin-α5 as compared to empty treated mice demonstrating tolerogenic reorganization. At a cellular level, we tested how MP treatment alters the formation of B cell microdomains - germinal centers (GCs). GCs were inhibited in treated and CNS-draining LNs in MOG/Rapa treated mice relative to empty treated mice in early and peak disease. This work engineers local LN microenvironments to support a desired immune outcome. A similar approach could be taken to reverse suppressive LN conditioning in cancer and support tumor clearance. At this conference, I will meet top researchers in the field. I am excited for this opportunity to receive expert feedback to improve my research and to expand my network as I look for a post-doctoral position.

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Continual systemic Treg recruitment to tumors mediate local immunosuppression

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Anti-cancer immunity is predicated on the ability of leukocytes to migrate into tumors. Once leukocytes are recruited, they undergo substantial reprogramming, including the development of T cell dysfunction, a process which restricts the ability to harness T cells to eliminate cancer. A major limitation in our understanding of cancer immunology is the ability to distinguish recently recruited leukocytes from those residing in the tumor for an extended period of time. Here, we developed an intravascular antibody technique to label circulating leukocytes before their arrival in tissues, enabling unprecedented resolution into the kinetics of recruitment. Using this approach in a model of lung adenocarcinoma revealed the dynamic recruitment of both innate and adaptive leukocytes, with a significant degree of migration occurring throughout tumor

progression for multiple immune cell lineages. In particular, regulatory T cells (Tregs) were recruited more robustly than tumor-specific CD8+ T cells. Treg recruitment was dependent on the integrins CD11a and CD49d. Antibody blockade of CD11a/CD49d tipped the immunological balance, driving an improved anti-tumor CD8 T cell to Treg ratio and decreased tumor burden. Our data highlight the relevance of constructing a precise temporal map of leukocyte recruitment into tumors, and suggests that targeting leukocyte migration could be effective for improving immunotherapy.

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Craters on the melanoma surface serve as hubs for CD8+ T cells

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While CD8+ T cells presence at the tumor border indicates immune response, their distribution, movement, and behavior are challenging to describe using 2D immunohistochemistry. Using a water flow system that keeps adult fish alive under a 2-photon scope, we applied 3D, time-lapse imaging for over 15 hours to track CD8+ T cells behavior on the surface of BRAF(V600E) derived melanoma in vivo. We followed CD8+ T cell infiltration using a novel CD8 reporter zebrafish that detects CD8+ T and dendritic cells (DCs), key players in cancer immunity. We found that rather than randomly surveying the tumor, CD8+ T cells preferentially aggregate in melanocyte-lined

pits on the tumor surface, resembling craters. The craters are sites of melanoma recognition and activation for the CD8+ T cells. CD8+ T cells travel to the craters from the venules containing tumor edge and interact with melanoma cells on the crater walls for hours before moving to the next crater or entering the tumor. CpG ODN injections and TGF-β inhibition, immunotherapies currently in clinical trials, induced increased infiltration of CD8+ T cells accompanied by crater enlargement. The CD8+ T cells preferentially accumulated in craters and were activated. Presence of multiple CD8+ DCs within craters, some of which clustered with CD8+ T cells or engaged in antigen uptake, as seen by time-lapse imaging, also indicated that the craters can serve as sites for antigen presentation to CD8+ T cells. In human melanoma, we identified craters at two main locations: the melanoma border and breaching from perivascular spaces. These craters harbor dysfunctional, proliferating CD8+ T cells, previously recognized as tumor-reactive T cells. As in the zebrafish, increased CD8+ T cell infiltration was accompanied by increased crater number. Taken together, we identified novel pathological features facilitating T cell infiltration in tumors. The craters are indicative of T cell infiltration and can be used to assess prognosis and immunotherapy outcome and may lead to new treatment approaches to increase T cell infiltration efficiency following immunotherapy.

Defining and Using Immune Archetypes to Classify Cancer

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Cancers display significant heterogeneity with respect to the tissue of origin, driver mutations, and other features of the surrounding tissue. It is likely that individual tumors engage common patterns of the immune system—here “archetypes”—creating prototypical non-destructive tumor immune microenvironments (TMEs) and modulating tumor-targeting. To discover the dominant immune system archetypes, the University of California, San Francisco (UCSF) Immunoprofiler Initiative (IPI) processed 364 individual tumors across 12 cancer types using standardized protocols. Computational clustering of flow cytometry and transcriptomic data obtained from cell sub-compartments uncovered dominant patterns of immune composition across cancers. These archetypes were profound insofar as they also differentiated tumors

based upon unique immune and tumor gene- expression patterns. They also partitioned well-established classifications of tumor biology. The IPI resource provides a template for understanding cancer immunity as a collection of dominant patterns of immune organization and provides a rational path forward to learn how to modulate these to improve therapy.

Defining the fate of egressing tumor-specific T cells and role in regional LN metastasis

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The success of immunotherapy has shifted the paradigm of cancer treatment, however, in many cases mechanisms of innate or acquired resistance continue to limit response. We recently demonstrated that one of the mechanisms of resistance to immunotherapy is the egress of functional CD8+ T cells from the tumor via tumor-associated lymphatic vessels. The probability of T cell exit was determined by the quantity and quality local antigen encounter, and we found that egressing T cells were both antigen-specific and functional. Although this work demonstrated that the enhanced retention of these egressing CD8+ T cells could improve response to immunotherapy, it also raised important questions; What is the long-term fate of egressing T cells and may they functionally contribute further to tumor progression? Here we test the hypothesis that tumor-specific T cells egressing from the tumor differentiate into long-lived populations within the lymph node (LN), that may initially protect against regional metastasis and can be reinvigorated by immunotherapy. To test this hypothesis, we use Kaede-tg mice, a photoconvertible model that allows us to track endogenous leukocyte populations as they migrate from the tumor microenvironment (TME) to the tumor draining LN. Exposure of the tumor to 420nm light induces conversion from green to red fluorescence, allowing for tracking of endogenous antigen-specific T cells for up to 24 hours. YUMMER1.7 murine melanomas were implanted into syngeneic Kaede-tg C57Bl/6 hosts and tumors were photoconverted 21 days post implantation. We found that T cells, both CD4+ and CD8+, frequently exit the TME via afferent lymphatic vessels. To profile the transcriptional states of egressing CD8+ T cells we sorted CD44+ cells from the tumor and photoconverted cells from the LN and submitted them for scRNAseq. Pseudotime analysis of intratumoral CD8+ T cells predicted a gradient of migratory potential with cells expressing Tcf7, Sell, and Cxcr4 exhibiting the highest likelihood of egress while cells expressing Pdcd1, Lag3, Cxcr6, and Havcr2 were most

likely to be retained. Consistent with this prediction, T cells that recently exited the TME were enriched for Tcf7 expression but exhibited additional transcriptional heterogeneity. In particular, we found clusters of exiting CD8+ T cells that scored for stem (e.g. Slamf6, Tox, Tcf7, Klf2, Cxcr5) and resident memory (e.g. Cxcr6, Il7r, Itgae, Cd69) transcripts. We validated the presence of a CD8+TCF1+PD-1+ stem-like egressing T cell population by flow cytometry and observed the presence of a CD8+CD69+CD62L- resident-like population in the tumor-draining LN. Our data therefore supports an emerging model in which egressing CD8+ T cells differentiate as they enter the LN and may feed into resident stem and memory populations. Ongoing work will determine the extent to which these populations contribute to metastatic control and response to immunotherapy.

Determinants of CD8+ T cell behaviour within hepatocellular carcinoma

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CD8+ T cells play a crucial role in controlling liver tumours, such as hepatocellular carcinoma (HCC) however we have only limited knowledge of the precise dynamics of their interactions with hepatic parenchymal and non-parenchymal cells at the single-cell level.

Previous work from our laboratory, demonstrated that in the context of HBV-expressing hepatocytes circulating effector CD8+ T cells (Teff) perform their immune surveillance function recognizing the antigen and kill virus-expressing hepatocytes extending cytoplasmic protrusions through endothelial fenestrations while still within liver sinusoids. Here we dissected whether

similar or different mechanisms govern the capacity of Teff to home, migrate, recognize the antigen, and exert effector function within HCC.

The first effort to dissect the project was the establishment of a new murine model of spontaneous HCC in which just the transformed hepatocytes express a nominal antigen, the oncogene SV40 large T antigen (TAg), and a fluorescent protein. We were able to obtain mice that develop spontaneous HCC lesions, highly proliferating and spread in a normal liver parenchyma. After in vitro effector differentiation and adoptive transfer of TAg-specific Teff in tumor-bearing mice, we observed that just some mice respond to the cytotoxic activity of the transferred cells, eliminating partially or completely the tumor, while in other mice the adoptive transferred cells have no beneficial effect on the tumor elimination. Thus, using a mathematical approach, we managed to pick the lesion volume as the fundamental parameter to predict the fate of each single HCC lesion: we called “responders” (R) the HCC lesions that are responsive to the cytotoxic activity of the TAg-specific Teff, with a single HCC lesion volume <10 mm3, while we called “non-responders” (NR) the HCC lesions that are not responding to the cellular therapy and they have a single HCC lesion volume > 100mm3.

We then studied the determinants that confer the therapeutic activity to the TAg-specific Teff in R and the ones that dampen the therapeutic activity in the NR lesions. We observed a reduced early tumor infiltration of TAg-specific Teff , single cell motility and activation in NR lesions compared to the R, suggesting that there are some hemodynamical and/or environmental features intrinsic in the R and NR responsible for the effective or un-effective tumor elimination of the adoptive transferred TAg-specific Teff. Our main finding was that the vessel phenotype was completely different in R and NR: the HCC volume gain correlates with more neovascularization, capillarization and a strong reduction of liver sinusoidal endothelial cells (LSECs).

Overall, we observed that although Teff have the capacity to induce tumor regression, their ability to clear advanced tumors is limited. Our data support the hypothesis that some anatomic, hemodynamic, and environmental features acquired by each individual HCC lesion, during their growth, can influence their responsiveness to the Teff killing. The innovative nature of our work will elucidate new mechanisms whereby Teff fail to exert their immune function and cytotoxic activity in tumorigenic liver.

Development of a Personalized Neoantigen Specific TCR Discovery Platform

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Tumor-infiltrating lymphocyte (TIL) therapy can mediate tumor regression in a range of solid can most notably in melanoma. However, its wider application and efficacy has been limited by the lo frequency and exhausted phenotype of tumor-specific T cells in the final product. Here, we devel a personalized, neoantigen-specific TCR discovery platform that will enable engineering of multip TCRs into autologous peripheral blood T cells. This allows for the generation of a fitter T cell prod with a high frequency of tumor-reactive TCRs of defined specificity.

Our platform first identifies tumor-specific mutations and TIL-derived TCR repertoires from non-viable tumor specimens using next-generation sequencing (NGS), which are subsequently recreated using synthetic biology technology. The synthesized TCR libraries are expressed in reporter T cells, whereas neoantigen libraries are engineered in autologous APCs. Following coculture of these cells, activated and non-activated T cells are separated, followed by neoantigen-specific TCR identification using NGS-based analysis.

We validated the high sensitivity and specificity of this platform by successfully identifying multiple TCRs and their cognate neoantigens from high tumor mutational burden (TMB) cancers including melanoma. Notably, we show that the platform is agnostic to the type of mutation and HLA class restriction. Importantly, neoantigen-specific TCRs can also be isolated from a panel of low TMB microsatellite-stable colorectal cancers, underscoring the pan-cancer potential of this approach.

We commenced applying this approach in a clinical study in which patients will be treated with functional autologous T cells engineered with neoantigen-specific TCRs of defined specificity and composition.

Development of a production process to generate CD8+ T cell-enriched tumor infiltrating lymphocyte (TIL) products with increased cytotoxic potential for the treatment of patients with solid cancers

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Background: Treatment with autologous tumor infiltrating lymphocytes (TILs) can induce remarkable clinical responses. The absolute numbers of CD8+ T cells in TIL products have been shown to correlate with clinical responses upon TIL therapy. With the current production process, the numbers of CD8+ T cells in the TIL products vary greatly between patients. By using a targeted cytokine that preferentially activates CD8+ T cells, we aimed to increase the cytotoxic potential of the TIL products.

Methods: A cis-targeted CD8-IL2 molecule (Asher Biotherapeutics) was used to promote CD8+ T cell outgrowth from tumor digests. In the rapid expansion protocol (REP), TILs were subjected to polyclonal stimulation using anti-CD3 antibodies (OKT-3) or anti-CD3/CD28 polymers (TransAct™) in the presence of CD8-IL2. The standard 'young TIL' production process using high dose IL-2 and OKT-3 was used as a standard comparison. TIL product composition, T cell differentiation phenotype and tumor-reactivity was assessed by flow cytometry, ELISA and cytokine bead arrays.

Results: 7 tumors (5 melanoma, 1 cervical carcinoma and 1 endometrial carcinoma) were subjected to enzymatic digestion. At the end of the REP phase, TIL products cultured with CD8-IL2 contained 98% (range 53-99%) CD8+ T cells, compared to 74.7% (range 35-84%) for products cultured with conventional IL-2. T cell differentiation phenotypes of the TIL products generated with CD8-IL2 were similar to the standard production process, showing that the improved expansion and CD8+ T cell enrichment did not come at the expense of a more exhausted phenotype. Upon restimulation with tumor digest clear anti-tumor reactivity of the TIL products could be demonstrated based on IFN-y production and tumor cell kill.

Conclusion: This study shows that CD8 cis-targeted IL-2 can be used to generate TIL products mainly comprising CD8+ T cells, thereby potentially improving cytotoxic potential and therapeutic efficacy.

Development of a T cell receptor-based therapy targeting phosphopeptides for hematologic malignancies

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Tumor antigen-specific T cells can mediate durable remissions of otherwise refractory tumors provided the correct tumor antigen is targeted. Ideal antigens are characterized by differential tumor presentation and recognition as “non-self” by the immune system. Examples of antigens shared by tumors include neoantigens derived from RAS or p53 mutations. Phosphopeptides are an emerging class of mutationally-independent tumor antigens selectively presented on solid and liquid tumors and seen as non-self by virtue of their phosphate moiety. We sought to define new phosphopeptide tumor antigens and develop a phosphopeptide T cell receptor-based (TCR) therapy.

Based on prior work indicating differential presentation of phosphopeptides on EBV-transformed cells identical to phosphopeptides presented by leukemic cells, we initially performed HLA class I and II immunoprecipitation on 6 EBV-transformed cells, 1 healthy B cells, and 1 EBV+ lymphoma. We further mined all public phosphopeptide datasets spanning 123 samples, primarily lymphomas, leukemias, and meningiomas. To verify the HLA binding of selected phosphopeptides, we performed stabilization assays in TAP-deficient cells as well as molecular docking. To survey donor immune repertoires for phosphopeptide-specific T cells, we used combinatorial dextramer staining across multiple HLA alleles to detect and isolate phosphopeptide-specific T cells and employed IFNg ELISpot to assess their phosphopeptide-specific function. To recover TCRs specific for phosphopeptides, we used RT-PCR and 3’ index barcoding to identify TCR genes.

Our mass spectrometry analysis combined with public data mining yielded 2,466 unique class I and II phosphopeptides. Focusing on the serotypes HLA-A3 and -A11, we found 772 unique phosphopeptides presented by A3 or A11. We selected 5 phosphopeptides presented by multiple hematologic tumors and EBV-transformed cells, but not healthy cadaver tissue, that were derived from genes required for lymphoma and leukemia cell survival. Using HLA stabilization assays in TAP-deficient cells and molecular docking, we found that they all capably stabilized HLA-A3, but did not stabilize HLA-A3 to a greater extent than their unphosphorylated counterparts. This finding contrasts with previous reports of HLA-A2-presented phosphopeptides. Despite their equivalent binding, the phosphopeptides still adopt conformations distinct from their unmodified counterparts as determined by molecular docking. Using phosphopeptide-HLA dextramers, we could detect specific T cell responses to phosphopeptides presented by A0201 and A1101 that mediate phosphopeptide-specific IFNg responses in donors expressing these alleles. We were also able to generate T cells specific to the phosphopeptides presented by A0301 and C0701 from allogeneic donor repertoires without promiscuous binding to A0301 or C0701 dextramers complexed with irrelevant peptide. Analysis of C0701 phosphopeptide-specific T cells yielded 11 unique TCRβ and 17 unique TCRα clonotypes, with a preference for TRBV20-1.

In conclusion, we demonstrate that a series of phosphopeptides presented by prevalent HLA alleles constitute tumor antigens differentially expressed by lymphomas and leukemias, representing attractive targets for cancer immunotherapy. Furthermore, sourcing T cells from the allogeneic repertoire allowed us to recover TCRs specific for phosphopeptides presented by HLA alleles other than A0201. We are currently assessing the antitumor activity of effector cells bearing phosphopeptide-specific TCRs.

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Development of designer bacterial nanoparticles for cancer immunotherapy

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In recent years the world of oncology is shifting towards personalized therapies. A potential candidate comprises bacterial derived nanoparticles called protein bodies (PBs). These seem optimally suited for multi-epitope vaccine delivery and cancer immunotherapy. We previously demonstrated that PBs are potent inducers of DC maturation. Also, DCs were shown to properly internalize, process and present antigens administered as PBs to drive strong antigen-specific CD8+ and CD4+ T cell responses (*Schetters et al. (2020) Cell Mol Immunol. 17(4):415-417.*) Here, we further studied the efficacy of PBs *in vivo* using a B16-OVA mouse tumor model. Interestingly, mice immunized with particles containing the ovalbumin (OVA)-derived OT-I and OT-II epitopes showed no tumor outgrowth, compared to immediate tumor take in unvaccinated counterparts, demonstrating the ability of PBs to drive responses that impair tumor growth. Moreover, the efficacy in a human setting was analyzed using PBs comprising epitopes of the human melanoma antigen gp100. These particles induced strong moDC maturation and showed effective uptake by moDCs resulting in strong antigen-specific CD8+ T cell responses. Current research is aimed at tailoring targeting and immune responses to further optimize efficacy of the PB platform. Taken together, this research demonstrates the potential of PBs for use in therapeutic cancer vaccination.

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Discovery of a Molecular Clock that Controls CD8+ T Cell Function and Exhaustion

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During cancer and chronic viral infections, the persistence of antigen progressively causes CD8+ T cells to differentiate into a dysfunctional PD1+ “exhausted” state, with reduced production of inflammatory cytokines relative to effector cells that form during acute infections. Antigen and costimulation signals activate kinase cascades to induce distinct T cell transcription programs, but how T cells distinguish acute and chronic signals to program the exhausted or effector transcriptional states remains poorly understood. We found that members of the protein kinase C (PKC) family function together as a “molecular clock,” sensing acute or chronic agonism to drive distinct transcriptional programs. Continuous stimulation of PKC is sufficient to induce many features of T cell exhaustion, including a loss of production of the cytokines IFNγ and TNF, and an increase in the expression of GzmB. PKC agonism triggers other hallmarks of terminal exhaustion, including altered expression of genes in the AP-1 transcription factor family and upregulation of the exhaustion regulator TOX. Mechanistically, CD8+ T cells express several different PKC proteins, and chronic agonism of PKC leads to degradation of multiple family members and selective maintenance of only one PKC protein, PKC-η. This “PKC switch” alters the downstream signaling cascade to support the transcriptional reprogramming of T cells into a terminally exhausted state. Remarkably, chronic agonism of PKC even in the absence of antigen is sufficient to induce elements of the terminal exhaustion gene expression program, illustrating how the different forms of PKC function together as a signal transduction nexus during T cell exhaustion differentiation. In summary, continuous signaling through PKCs causes changes in the output from these kinases initially at the protein level, driving transcriptional changes among PKC targets in the AP-1 transcription factor family and thus allowing further widespread transcriptional and functional changes that characterize T cell exhaustion.

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Dissecting the role of the CD4 T cell-licensing of cDC1s for the generation of optimized anti-tumor CD8 T cell responses

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Conventional type 1 Dendritic Cells (cDC1s) are specialized in antigen (Ag) cross-presentation for CD8 T cell priming and play a key role in the cytotoxic T cell (CTL) response against cancer. For effective CD8 T cell priming, cDC1s are licensed by CD4 T cells that recognize Ag on MHC class II (MHC-II) molecules presumably presented by the same cDC1. CD4 help is in part mediated by CD40 signaling, which acts to enhance DC maturation and survival, but whose mechanisms and spatiotemporal regulation remain incompletely understood.

As a new approach to understanding the mechanistic basis of CD4 help, we compared two RNA-Lipoplex (RNA-LPX) vaccines that express a single MHC-II-restricted Ag either tumor-specific (neoAg) or, tumor irrelevant. RNA-LPX is injected intravenous and is taken up by professional antigen-presenting cells, including cDC1s, in the spleen and other lymphoid organs.

While both vaccines induced CD4 T cell responses and cDC1 maturation, including upregulation of CD40 expression, only the tumor-specific vaccine led to tumor rejection, tumor-specific CD8 T cell accumulation at the tumor site and cDC1 expansion and enhanced maturation in spleen. We found that cDC1s are required for vaccine-induced neoAg-specific CD4 T cell priming and tumor-specific vaccine efficacy. CD4 T cell responses elicited against non-tumor Ag were insufficient for generating anti-tumor CTL responses.

It is likely that vaccine-induced neoAg-specific CD4 T cells are primed by cDC1s and license the later for inducing CD8 T cell-mediated tumor rejection. In addition, the induction of any CD4 T cell response and CD40 expression in mature cDC1s alone appeared insufficient for licensing cDC1-mediated efficient anti-tumor CTL responses suggesting that other unknown helper mechanisms from tumor-specific CD4 T cells only are required. We are currently investigating these additional mechanisms using both *in vivo* and cell culture approaches.

This model will provide fundamental insight to elucidate the activation and transcription patterns in cDC1s following their licensing by tumor-specific CD4 T cells as well as define whether the licensing of cDC1s is also required to maintain CTL responses *in situ*. Finally, this strategy may also identify new therapeutic opportunities designed to enhance endogenous anti-tumor immune responses

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Dual-targeting cancer antigens and tumor microenvironment TGF-β with CAR-macrophages

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As macrophages are natural invaders into the tumor microenvironment, endowing macrophages with chimeric antigen receptor (CAR) is a tempting approach to target cancers. TGF-β is a major immunosuppressive cytokine that is overexpressed in the TME and limiting the effectiveness of adoptive cellular therapy. Here, we engineered CAR-macrophages to serve as a "Trojan horse" to deliver anti-TGF-β blocking scFv into the TME. We genetically targeted CAR and anti-TGF-β scFv cassettes in human pluripotent stem cells as a robust source of CAR-macrophages. We validated that anti-TGF-β blocked the SMAD signal in a reporter cell line. We anticipate that TGF-β scFv secreted by CAR-M could reinvigorate anti-tumor immune cells such as T cells, and natural killer cells that are originally suppressed by TGF-β. A dual CAR-macrophage system could actively eat up the tumor and remodel the immunosuppressive TME to a pro-inflammatory one, enhancing both innate and adaptive immunity.

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Effector T Cell Phenotypes and Cellular Communication Driven by Tregs Might Hold Key to Influencing CAR T Effectiveness in Multiple Myeloma

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Autologous chimeric antigen receptor T (CAR-T) cell therapies show significant clinical activity against hematologic malignancies including multiple myeloma (MM). A myriad of factors influences the effectiveness of this treatment modality. Fitness of a CAR-T cell product is an important factor affecting T cell engraftment and persistence, a pre-requisite for effective therapy. In an effort to deconstruct the cellular heterogeneity of CAR-T cell products and explore

relationships between T cell states within the cellular product, gene expression and CAR-T product following adoptive transfer, we performed single cell RNA-sequencing (scRNA-seq) on products from a phase I trial of BCMA-specific CAR-T cells in relapsed/refractory MM patients that included cohorts receiving a similar CAR-T cell dose with and without preconditioning with cyclophosphamide.

Method: scRNA-seq was performed on 25 unique products with an average of 8823 cells captured per product at an average depth of 40779 reads per cell. High resolution annotation was complicated by the artificial environment of CAR product manufacturing process which disrupts natural T cell RNA expression patterns. This was overcome by using stochastic inference modeling with expectation maximization to optimize its parameters and provides a cell-type prediction for each cell. Differential gene expression (DGE) analysis was conducted by both Wilcoxon rank sum test testing and validated with an adapted generalized linear model for bimodal and/or zero-inflated single cell gene expression data. Gene enrichment analysis was performed by running transcriptomes with the Molecular Signatures Database v7.4. Cell to cell communication utilized the KEGG signaling pathway maps and curated lists of interactions from the literature. The intercellular communication probability was estimated on the DE genes before statistically significant intercellular communications were calculated by a permutation test, with dominant senders, receivers, mediators, and influencers identified using graph theory. Patients were grouped in two ways, first with products that showed increased engraftment as defined by higher than median peak blood vector copies per cell and second classification-based response of partial response (PR) or greater with combined progression-free survival (PFS) of greater than 300 days.

Results: DGE analysis found 205 genes that were up or down regulated between the High vs Low engraftment phenotypes and 180 genes between the PFS groups. CAR-BCMA expression was increased in High engraftment groups which has not previously been shown in CAR sorted cells independent of vector copy number. There was a shift in more effector T cell signatures in the Low engraftment group. Within both CD4+ and CD8+ T cell subsets, transcriptional pathways associated with the RXF5/RFXAP/RFXANK transcriptional activator complex and the IL-2/STAT5 signaling were identified as upregulated in the High engrafted products. Cell to cell communication analysis for secreted signaling and cell to cell contact reinforced activation communication above but also revealed CD4+ Treg specific signaling with both stimulatory and inhibition, as well as cytokine interactions.

Conclusion: This study reveals potential cell to cell communication that could be augmented in the manufacturing stage to create product with a greater chance of engraftment as well as identifies potential necessary, but not sufficient, gene networks linked to higher PFS.

Elucidating mechanisms of T cell exclusion in melanoma

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Immunotherapy represents a paradigm shift in cancer treatment. Immune checkpoint blockade (ICB) and adoptive transfer of engineered T cells have recently produced durable responses in many patients for whom previous therapies were ineffective, demonstrating the essential role of the cancer-immunity interface in patient outcomes. However, not all patients respond to available immunotherapies, and some who initially respond become resistant. Immune cells in the tumor microenvironment (TME) play a role in the response to checkpoint therapies; it is known that a high density of CD8 T cells correlates to better outcomes. We aim to clarify how tumors exclude T cells from their microenvironment which will likely reveal novel principles of successful cancer immunotherapy.

Part of the barrier to understanding the mechanisms of T cell exclusion is the lack of robust in vivo models that are representative of the diversity of human cancer. We developed models of T cell exclusion using human melanoma cell lines in a humanized mouse. Specifically, these models rely on humanized 'MISTRG' mice, which are transplanted with human CD34+ hematopoietic stem and progenitor cells to give rise to human blood cells, including T cells, macrophages and dendritic cells. After introducing melanoma cells subcutaneously, we showed that five human melanoma cell lines exhibit distinct T cell infiltration patterns: immune desert (lacking T cells), immune-excluded (T cells at tumor border) or immune-infiltrated (T cells in tumor parenchyma). Our results are consistent with the distinct patterns characterized in patient biopsies and suggest that T cell positioning in the TME of each tumor line is a tumor-intrinsic property.

Because infiltration appears to be instructed by the tumor cells, we used bulk RNA sequencing to identify differentially expressed genes between T cell-infiltrated (AKA hot) versus -excluded/desert (AKA cold) tumors. We found unique gene signatures and were able to create a score of how "hot" or "cold" each tumor cell line was. We applied this score to an outside dataset of 48 melanoma lines and obtained eight lines that either highly expressed the hot or cold gene sets. We implanted each of the eight lines into the MISTRG model and found that our gene signatures were indeed predictive. The four predicted "cold" tumors had very few T cells in the stroma and virtually no T cells in the tumor parenchyma, while the predicted "hot" tumors had T cells in the tumor and higher overall T cell densities in the tumor and stroma.

Our results demonstrate that T cell infiltration is directed by the tumor cells and that specific gene signatures can predict the pattern of infiltration. Our experimental platform enables us to go beyond correlative studies and identify causality between transcriptional patterns and in vivo function across diverse human tumors.

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Elucidating the principles that govern the therapeutic window of HER2-directed CAR T cells

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Paucity of tumor-specific targets presents an obstacle to developing safe and effective chimeric antigen receptor (CAR) T cell therapy against solid tumors. Success in blood cancers was facilitated by the high and homogeneous expression of lineage-restricted antigens on tumor cells and their absence on vital tissues. However, cellular therapy in solid cancers has exhibited less efficacy and led to unpredictable toxicities due, in part, to off-tumor on-target antigen recognition. Because potential autoimmune toxicities from novel CAR T cell therapies remain difficult to predict *a priori*, there is a need for developing robust preclinical models to study the therapeutic window of CAR T cells.

We developed a syngeneic model of anti-murine HER2 CAR T cell therapy and examined CAR-intrinsic factors that govern their safety profile. A library of murine HER2-specific monoclonal antibodies with a wide range of affinities was generated and used for immunohistochemical analysis of normal mouse tissue. Akin to humans, mice express HER2 on the epithelial tissues of the gastrointestinal tract, endometrium, and airways, which renders these tissues potentially vulnerable to aberrant targeting by HER2-directed CAR T cells. Employing mHER2.CARs derived from low-affinity scFvs did not confer off-tumor on-target toxicity, and CAR T cells infiltrated HER2-overexpressing tumors and transiently controlled tumor growth while sparing normal tissue. Increasing the binder affinity markedly improved CAR T cell recognition of low-HER2 expressing tumor cells *in vitro* but failed to improve anti-tumor function *in vivo*. Moreover, high-affinity CAR T cells elicited overt, moderate-to-severe toxicity to normal tissues and death. The observed toxicity was on-target, independent of tumor burden, and correlated with the extent of systemic proliferation of CAR T cells post infusion. Our findings define a syngeneic mouse model in which the CAR affinity, anti-tumor activity, and potential off-tumor on-target toxicity can be methodically investigated to define the therapeutic window of CAR T cell therapy against a clinically relevant target.

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Endogenous IL-27 signaling is essential for maintenance of tumor neoantigen-specific T cells and therapeutic IL-27 agonism controls established tumors in mice

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Cytotoxic CD8+ T cells are essential effectors in antitumor immune response and are key players of currently approved cancer immunotherapies. We identified IL-27, a cytokine that has been described to have both pro and anti-inflammatory effects in various immunological settings, as being highly correlated with a CD8+ T cell cytotoxic signature in diverse human and murine tumor types. Endogenous IL-27 improved CD8 T cell maintenance and activity, preventing exhaustion of tumor-infiltrating T cells. Moreover, sustained systemic overexpression of IL-27 was well tolerated, induced tumor regression in inflamed murine tumors, and drove a cytotoxic program in neoantigen-specific CD8+ T cells, in both the draining lymph nodes and tumor. The beneficial effect of IL-27 on CD8+ T cell cytotoxic activity was associated with an increased CD8T cell maintenance. In addition, IL-27 agonism synergized with PD-L1 blockade to induce regression of multiple tumor models that were resistant to single-agent anti-PD-L1 immunotherapy. Interestingly, in combination with PD-L1 blockade, IL-27 agonism restricted to the tumor microenvironment was sufficient to promote both CD4+ and CD8+ T cell responses. Overall, we conclude that endogenous IL-27 is essential for maintenance of anti-tumor immunity, and that IL-27 agonism can improve anti-tumor T cell responses, alone or in combination with existing immunotherapies.

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Enforcement of exhausted T cell epigenetic fate by HMG-box transcription factor TOX

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T cell exhaustion is one of the major barriers limiting efficacious anti-tumor therapy. Exhausted CD8 T cells (T_{EX}) develop following persistent antigen stimulation and are characterized by a unique epigenetic state, expression of PD-1 and other inhibitory receptors, dampened effector function, and limited capacity to control disease. Though checkpoint blockade temporarily improves T_{EX} function, the underlying epigenetic landscape of T_{EX} remains largely unchanged and these "reinvigorated" T_{EX} revert to less effective antitumor T cells. Thus, a better understanding of the epigenetic determinants and flexibility of T_{EX} fate commitment should reveal novel therapeutic opportunities for tuning T cell differentiation in cancer and other diseases. Our lab and others recently identified the HMG-box transcription factor TOX as an essential transcriptional and epigenetic initiator of T_{EX} lineage differentiation. Here, we investigated whether sustained TOX expression was required to maintain the T_{EX} epigenetic identity and fate inflexibility. Induced TOX ablation in committed T_{EX} reduced T_{EX} numbers and impaired expression of PD-1 and other canonical T_{EX} inhibitory receptors. This effect of TOX loss appeared to be driven by altered T_{EX} proliferation and survival. Accordingly, Bim knockdown numerically rescued TOX-deficient T_{EX}; yet, PD-1 loss and other key T_{EX} phenotypic differences were retained. This incomplete rescue alluded to a global role for TOX in enforcing T_{EX} differentiation, beyond directly regulating T_{EX} survival. Indeed, single-cell RNAseq and ATACseq revealed that TOX was required to maintain transcriptional modules of mitochondrial function, protein synthesis, terminal differentiation, and epigenetic factor expression. These gene expression differences correlated with loss in chromatin accessibility. Furthermore, removal of TOX-deficient T_{EX} from a chronic environment enabled their partial reprogramming into the more functional effector lineage, thus identifying TOX as one of the epigenetic barriers that constrain the fate flexibility of T_{EX}. Together, these findings suggest that TOX transcriptionally and epigenetically enforces critical components of the T_{EX} program, and that TOX manipulation provides an avenue for rewiring T_{EX} identity. By improving molecular understanding of the role of TOX in enforcing T_{EX} identity and in constraining T_{EX} fate reprogramming, this study will inform future immunotherapies that seek to re-engineer T_{EX} into customized differentiation states with amplified potential for tumor control.

Establishing the relevance of mouse models to the Human tumor immune archetypes

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The past years have highlighted the plurality of immune states found in the tumor microenvironment (TME) across cancers. In a recent study, we leveraged compositional and transcriptomic data from hundreds of surgical specimens across tumor types to uncover 12 dominant patterns of immune composition, named archetypes (Combes, Samad et al., Cell 2022). These archetypes range from good prognosis immune-rich profiles biased toward CD8 T cells and monocytes infiltration, to poor prognosis immune desertic tumors enriched for tumor-associated macrophages (TAM). While immune archetypes aid in tumor classification and survival stratification, their relationship to immunotherapies remains unclear. Expanding this classification in animal models is warranted to further study these relationships and develop tailored therapeutic targets.

To this end, we developed an immunoprofiling pipeline of mouse tumors combining CyTOF and scRNAseq to benchmark 15 commonly used mouse tumor models against the 12 human immune archetypes. We also profiled 6 of these models under conditions perturbing the host gut microbiota or age to establish the role of such fundamental perturbations on the TME.

By analyzing our cohort of 291 mouse tumor samples we observed that, despite displaying inter-model diversity, mouse models fail to mimic the immune composition profiles found in cancer patients. This is vastly due to a lower T cell infiltration accompanied by a strong bias toward myeloid cells and particularly TAM, found at levels unseen in patients. While perturbating gut microbiota or age induce limited changes in TME composition that appear model-specific, these perturbations don't improve the modelling of human immune diversity in mouse tumors.

Nevertheless, we observed that immune cells infiltrating mouse tumors conserve gene expression features from their Human counterparts. Namely, tumor-associated T and myeloid cells express compartment-specific chemokines which overlap between species. Comparing inter-model to inter-archetype gene signature patterns also highlighted cross-species conservation, pointing at possible pairings between specific Human archetypes and mouse models at the gene expression level.

Overall, our work is establishing the relevance of the most commonly used mouse tumor models to the dominant TMEs observed in human solid tumors. It highlights the limited coverage of the Human compositional profiles by mouse models, and therefore clarifies the extent to which the community should rely on these existing mouse models to understand and manipulate cancer immunity in patients.

Ex vivo co-culture platform established to assess reactivity of tumor infiltrating lymphocytes to primary head and neck cancer cultures

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There is tremendous interest in the use of tumor infiltrating T cells (TILs) as a cancer therapeutic across a range of malignancies. Autologous TILs therapy has been reported to achieve objective response rates ranging from 20-50% for metastatic/recurrent solid cancers including melanomas, breast and liver cancers. However, there is still much unpredictability with regards to its efficacy, especially when extended across different centres. Hence, there is much potential to improve expansion strategies, establish better biomarkers and predictive assays in solid cancers with unknown tumor-associated antigens. Here, our aim was to extract TILs from tumors of non-viral associated head and neck squamous cell cancer (HNSCC) with minimal alteration to the original tissue structure, expand these using the Rapid Expansion Protocol (REP) (denoted as REP-TILs), and examine their phenotype and functional ability to target cancer cells. To do this, we treated tumor fragments with interleukin-2 for 14-21 days (initiation) and subsequently subjected the TILs to REP for another 14-21 days. Five to seven days after the initiation, cells extravasated from the tumor fragments and these were predominantly CD3+ T cells as confirmed by flow cytometry. Next, REP-protocol achieved a 62-948 fold expansion, and REP-TILs obtained through this methodology comprised >93% of T cells (CD3+) with a mixture of CD4+CD3+ (0.12%-98.6%) and CD8+CD3+ (1.01%-98%) T cells (n=6). Among the CD8+ population, we observed more than >97% expressed markers related to antigen-experienced/memory (CD45RO), while there was a diverse frequency that were exhausted/dysfunctional (CD57, LAG3, PD1, and TIM3) and tumor-specific (CD39, CD103 and CD137). When REP-TILs were co-cultured with patient-matched primary cancer cell cultures to assess T cell reactivity (n=5), two of five showed an increasing frequency of CD137+CD8+ T cells with increasing effector to target (E:T) ratio, compared to REP-TILs alone, indicating presence of tumor-specific T cells. Further

interrogation of the two REP-TILs showed that there was a higher frequency of CD137+, TIM3+ and CD39+, and lower frequency of CD57+ CD8+ T cells, when compared to the remaining three with little to no T cell reactivity. Blockade of HLA class I on cancer cells reduced the frequency of CD137+CD8+ T cells, whereas upregulation of HLA class I increased the frequency of these cells, indicating that the REP-TILs likely recognized cancer cells via TCR-HLA class I interactions. Real-time cell cytotoxicity analysis of these co-culture systems showed a similar trend of dose-dependent increase in cancer cell death as the E:T ratio ascends, and this was again reduced when HLA class I was blocked. Altogether, our results demonstrate REP-TILs obtained from non-viral associated HNSCC tumors retain anti-tumor recognition and cytotoxicity against cancer cells. Our data establishes an ex vivo platform that can be used to identify tumor-targeting subpopulations and further refine biomarkers of response, all of which can improve the use of TILs as a therapeutic in non-viral cancers.

FcγRIIB expressed on CD8 T cells limits responsiveness to PD-1 checkpoint inhibition in cancer

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Immune checkpoint blockade using Fc-containing monoclonal antibodies has emerged as a powerful therapeutic approach to augment anti-tumor immunity. We recently showed that FcγRIIB, the only inhibitory IgG-Fc receptor, is expressed on a subset of differentiated effector CD8+ T cells in mice and humans, raising the possibility that CD8+ T cell responses may be directly modulated by checkpoint inhibitor binding to T cell-expressed FcγRIIB. Here, we show that despite exhibiting strong proliferative and cytokine responses at baseline, human FcγRIIBpos CD8+ T cells exhibited reduced responsiveness to both PD-1 and CTLA-4 checkpoint inhibition as compared to FcγRIIBneg CD8+ T cells *in vitro* ($p<0.05$). Moreover, frequencies of FcγRIIBpos CD8+ T cells were reduced following treatment of human melanoma patients with nivolumab *in vivo* ($p<0.05$). This reduced responsiveness was FcγRIIB-dependent, because conditional genetic deletion of FcγRIIB on tumor-specific CD8+ T cells improved response to checkpoint blockade in a B16 and LLC mouse melanoma model ($p<0.01$). The limited responsiveness of FcγRIIBpos CD8+ T cells was also dependent on an intact Fc region of the checkpoint inhibitor, in that treatment with Fc-devoid anti-PD-1 F(ab) fragments resulted in a

significant increase in proliferation of FcγRIIBpos CD8+ T cells, without altering the response of FcγRIIBneg CD8+ T cells ($p<0.05$). Finally, blocking FcγRIIB in the context of PD-1 blockade significantly improved anti-tumor CD8+ T cell responses in B16 melanoma, Lewis lung carcinoma, and MC38 colon adenocarcinoma mouse models ($p<0.05$, $p<0.001$, $p<0.01$). These results illuminate an FcγRIIB-mediated, cell-autonomous mechanism of CD8+ T-cell suppression which limits the efficacy of checkpoint inhibitors during anti-tumor immune responses *in vivo*. The data presented here support the novel conclusion that CD8-expressed FcγRIIB is both a factor to consider in the development of therapeutic antibodies, and a new potential target for immunotherapeutic intervention.

Functional studies of IPMN microbiota in pancreatic cancer pathogenesis

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Background: Emerging research suggests gut microbiota may play a role in pancreatic cancer initiation and progression. Recently, our studies reported that cultivated pancreatic tumor microbiome from intraductal papillary mucinous neoplasm (IPMN), demonstrated pathogenic properties including intracellular hiding and induction of double-stranded DNA breaks upon co-cultivation with pancreatic cell lines (Gaiser et al. GUT 2019 and Halimi et al. Gut Microbes 2021). However, recent host cell-microbe interaction studies are limited to 2D monolayers, possibly they do not truly recapitulate the immediate spatial, cellular, and metabolic environment of highly complex tissues such as pancreatic cancer.

AIM: To study the bacterial pathogenic effect and invasiveness in normoxic and hypoxic conditions in the 3D spheroid model.

Methods: To mimic the pancreatic tumor environment, we have introduced the 3D spheroids model containing either normal healthy pancreatic cell lines in monospheroids, such as hTERT-HPNE, or hetrospecies hetrospheroids of pancreatic stroma (mPSCs) and cancer cell lines such as (Panc-1). Hypoxia is a common sign in solid and malignant tumor growth, we assessed the impact of the hypoxic and normoxic environment on bacterial invasiveness and pathogenesis by

staining infected spheroids with bacterial as well as DNA damage marker and used light sheet microscopy to gain a higher imagine depth in 3D with good spatial resolution. Immune recognition of extra- and intracellular microbiota is studied using several methods.

Findings:

Granulicatella adiacens and Enterobacter cloacae established consistent pathogenic effects in pancreatic healthy as well as in cancer spheroid. Moreover, we observed that in pancreatic cancer spheroid, and especially in hypoxic conditions bacteria are prone to infection and DNA damage. Immune recognition of intracellular- as well as extracellular microbiota is detected.

Conclusions:

It is reported that the presence of tumoral-associated bacteria in the pancreas is independent of the use of antibiotics prophylaxis. Our study will provide the opportunity to screen antimicrobial agents including immunotherapeutic approaches to reduce cancer risks that are related to the tumor microbiota.

Generation of functional macrophages from human embryonic organoids

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Single-cell characterization of embryonic development is a major benchmark of human developmental biology. Spatiotemporal analysis of stem-cell-derived embryos offers conceptual and technical advances in generation of cells wanted. Here, we defined gene expression landscape of human embryonic development with stem-cell-derived organoids. The human embryonic organoid (HEMO) recapitulated development of placenta, yolk sac, and trunk. Trophoblast-like tissues facilitated neural crest maturation via WNT signal. Hematopoietic tissues predominated HEMO with erythropoiesis, mekagaryopiesis, and myelopoiesis. Imaging-combined spatial transcriptomics defined the yolk sac erythro-megakaryopoietic niche. Vitronectin-integrin signaling remarked the yolk sac niche in HEMO consistent with human fetal yolk sac. Myelopoiesis populations contained common myeloid progenitors and monocytes, suggesting another wave of yolk sac hematopoiesis. We further maturated the monocytes with M-CSF into macrophages. The resultant macrophages responded to either pro- or anti-inflammatory cytokines, and phagocytosed hepatocellular carcinoma cells. Our functional macrophages from human embryonic organoids offer the platform to harness immune cells in cancer immunotherapy.

Genetic manipulations to improve intratumoral trafficking of adoptively transferred T cells

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Adoptive T cell transfer therapies, including chimeric antigen receptor (CAR)-T, have been successful in treating circulating B cell malignancies. However, efficient intratumoral infiltration of adoptively transferred cells remains a significant barrier to effectively treating solid tumors. One potential strategy to overcome failure of patient response is to enhance the ability to deliver genetically modified T cells to tumors. Identification of mechanisms contributing to T cell infiltration into tumors could lead to approaches that enhance existing therapies or support development of new therapeutic interventions. We recently performed an *in vivo*, genome-wide screen to identify T cell-intrinsic regulators of endogenous T cell intratumoral accumulation into solid tumors using the *Sleeping Beauty* (SB) transposon system. We generated a list of over 400 promising and novel candidates, including *AP-2 associated kinase 1 (Aak1*).

While others have performed CRISPR or shRNA loss-of-function screens with similar goals, the SB approach can also identify gain-of-function (or overexpression) mutations. To determine how our gene candidates, and particularly those gain-of-function candidates uniquely identified in the SB screen, compared with candidates from 5 independent loss-of-function-only screens, we implemented a rank aggregation algorithm to generate a single, integrated ranked list of all significant candidates in descending order of selection strength. Aggregated results yielded approximately 1500 T cell gene candidates whose biological functions are enriched in chemotaxis and many of which are not currently in therapeutic development. Importantly, *Aak1* remained the most strongly selected gene candidate. We hypothesize that *Aak1* represents a novel therapeutic target to improve T cell migration into solid tumors.

We have validated the most strongly selected gain-of-function candidates uniquely identified using the SB approach, including overexpression of truncated *AAK1 (AAK1^{DN125})*. Specifically, *in vivo* migration of adoptively transferred T cells into tumors, but not spleen or lymph nodes, was significantly increased when the T cells overexpressed *AAK1^{DN125}*. Further, *AAK1^{DN125}* expression improves selective T cell migration *in vitro* toward chemokine receptor CXCR3 ligands, but not CCR2, CCR5, or CCR7 ligands, suggesting there may be specificity for inflamed tissues. We are currently assessing impact of *AAK1^{DN125}* expression on T cell cytotoxic capability *in vitro* and examining therapeutic efficacy against solid tumors using *in vivo* CAR-T

models. Our data strongly support developing *AAK1*^{DN125} as a strategy to increase adoptively transferred T cell homing to the tumor microenvironment and expand the efficacy of adoptive T cell transfer therapies beyond circulating malignancies.

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Harnessing Epigenetic Therapies for Cancer Vaccine Efficacy

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Background: Cancer vaccines can induce robust anti-tumor T cell expansion yet have largely failed in clinical trials due to challenges in i) identifying widely expressed vaccine targets, ii) minimal target presentation on tumors due to low antigen or MHC expression, and iii) immunosuppressive tumor microenvironments (TME). Therapies targeting epigenetic modulators have recently been described to activate a 'viral mimicry' response in cancer cells: activation of transposable element transcription creates viral-like nucleic acids that are recognized by innate immune sensors, resulting in a type I interferon (IFN-I) response. Epigenetic drugs also have the potential to upregulate tumor antigens by remodeling the epigenetic landscape, including cancer testes antigens, tumor associated antigens, and TE-derived antigens. The FDA-approved DNA methyltransferase inhibitor azacytidine can induce a viral mimicry IFN-I response, upregulate Major Histocompatibility Complex (MHC) and increase CD4 and CD8+ T cell infiltration in melanoma. Yet, no studies have explored vaccination targeting epigenetic treatment-induced antigens (TIAs) in melanoma. This project identifies epigenetic drugs with innate and adaptive immunostimulatory properties, making them attractive candidates for future combination immunotherapy approaches.

Methods: B16 Blue cells express a SEAP reporter controlled by the ISG54 promoter, allowing high throughput screening of epigenetic drugs that induce interferon stimulated gene (ISG) responses. Knockout of MAVS and STING was used to identify which drugs elicited a response dependent on these key innate nucleic acid sensing pathways, indicating the presence of viral-like transcripts. To characterize the innate and adaptive immunomodulatory properties of drugs identified through screening, total RNA sequencing and immunopeptidomic mass spectrometry was performed, allowing differential expression of ISG and TE transcripts and antigen-MHC complexes.

Results: 65 total epigenetic drugs were screened, with 22 demonstrating a MAVS or STING dependent ISG response. Of these, 8 showed a high ISG response and synergized with IFNβ to induce upregulation of MHC. The DNA methyltransferase inhibitor azacytidine and the histone deacetylase inhibitor entinostat were further characterized, showing upregulation of ISGs and TEs by RNA sequencing. Azacytidine-treated cells also showed complete remodeling of the antigen repertoire presented on MHC, in particular demonstrating upregulation of key melanoma antigens Env2, Tyrp2, Pmel, and Melan-A.

Conclusions: We have established a screening approach to identify epigenetic drugs with innate and adaptive immunostimulatory properties. Further characterization of drugs identified through screening demonstrates that cells treated with azacytidine activate an innate IFN-I response, upregulate TE transcripts with the potential to encode antigens, and dramatically alter their immunopeptidome. This demonstrates that azacytidine, and other similar epigenetic drugs, have immunostimulatory properties that have the potential to help overcome barriers to cancer vaccine efficacy and augment immunotherapy response.

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High Avidity CD8 Tumor Infiltrating Lymphocytes Are More Exhausted and Less Responsive to Immune Checkpoint Blockade Than Their Low Avidity Counterparts

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The interaction of T cell receptors (TCRs) on T cells with MHC I-peptide complexes on cancer cells elicits the cytotoxic activity of CD8 T cells. Here we analyze how the strength of this interaction, or T cell avidity, shapes the exhaustion of CD8 TILs and dictates anti-tumor immunity. We developed a novel tetramer decay assay to isolate T cells based on their TCR avidities. We used this method to study low and high avidity CD8 TILs responding to the tumor antigen $PDPR^{MUT}$ of the murine sarcoma Meth A. Unbiased clustering of single cell RNA sequencing, TCR sequencing, and CITE-seq of these $PDPR^{MUT}$ -specific CD8 TILs revealed five clusters: Stem-Like ($LY108^{+}$*Tcf7*$^{+}$), Effector-Exhausted (Eff-Exh PD-1^{Low},$CX3CR1^{+}$) Terminally-Exhausted-1 (Term-Exh1 PD-$1^{+}TIM3^{High}$), Terminally-Exhausted-2 (Term-Exh2 PD-1^{High}*Tox*^{High}), and NK-Like (PD-1^{Low}, *Klr*^{High}). Low avidity T cell clonotypes had significantly more Eff-Exh (Student's t-test; $P< 0.005$) and significantly less Term-Exh1 (Student's t-test; $P< 0.05$) than high avidity T cell clonotypes. Trajectory inference of all cells, by scVelo, predicts that Stem-Like cells

differentiate into the Eff-Exh cluster that can differentiate into either Term-Exh1 or Term-Exh2. However, our data demonstrates Eff-Exh cells in low avidity clonotypes exclusively differentiate into Term-Exh1, while Eff-Exh cells in high avidity clonotypes differentiate into both Term-Exh1 and Term-Exh2.

We used flow cytometry to analyze $PDPR^{MUT}$-specific CD8 TILs with low or high avidity from 28 day-old Meth A tumors. There were significantly more $TIM3^{+}PD\text{-}1^{+}$ cells (Paired t-test; $P < 0.0001$) and $TOX^{+}PD\text{-}1^{+}$ cells (Paired t-test; $P < 0.05$) in the high avidity CD8 TILs than the low avidity CD8 TILs. We adoptively transferred 1,000 $PDPR^{MUT}$-specific low or high avidity CD8 T cells, or control CD8 T cells into tumor-bearing mice 10 days after tumor challenge. Low avidity T cells significantly improved the survival of mice (Mantel-Cox;$P = 0.0044$), while high avidity t cells did not do so (Mantel-Cox;$P = 0.7081$). These results establish a novel correlation between avidity, exhaustion and T cell-mediated tumor control in vivo.

Finally, we used flow cytometry to analyze the differential response of $PDPR^{MUT}$-specific CD8 TILs with low or high avidity from 28 day-old Meth A tumors to *in vivo* PD-1 or CTLA-4 blockade. Anti-PD-1 treatment increased the number of Stem-Like $PDPR^{MUT}$-specific TILs of low avidity (t-test; $P < 0.05$). Anti-CTLA-4 treatment increased the number of Stem-Like and Term-Exh1, while decreasing the number of Eff-Exh, low avidity $PDPR^{MUT}$-specific TILs (t-test; $P < 0.05$). No significant change in the phenotype of high avidity $PDPR^{MUT}$-specific TILs was observed after either PD-1 or CTLA-4 blockade *in vivo.* These data indicate that immune checkpoint blockade *in vivo* primarily acts on low avidity CD8 TILs.

High-throughput screening of synthetic TCR libraries reveals a transcriptional landscape of tumor reactivity and enables personalized polyclonal TCR-T cell therapy

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Background

Single-cell RNA-seq (scRNA-seq) has enabled TCR sequencing from thousands of T cells; however, the high cost of TCR synthesis makes it cost-prohibitive to screen the vast number of TCR sequences necessary to discover tumor-reactive clones. This severely hinders the ability to

develop personalized T cell-based therapies. To solve this problem, we developed two novel technologies to synthesize TCRs: one allowing for synthesis and cloning of thousands of TCRs in a pooled format, and the second which enables single-well synthesis of hundreds of TCRs in an arrayed format. These methods simplify the high-throughput experimental screening of hundreds to thousands of TCRs for tumor reactivity. We have applied these synthesis technologies to almost 40 individual human samples, generating and screening over 60,000 TCRs from TILs isolated from many solid tumor indications.

Methods

We isolated T cells from dissociated tumor samples (TIL) and then obtained the transcriptome and VDJ sequences using single-cell technology. Then we used our technologies to synthesize hundreds to thousands of TCRs and expressed them in normal donor peripheral T cells. These synthetic T cells were screened for tumor reactivity by co-culturing with autologous tumor or allogeneic cell lines expressing patient-matched HLAs. In the pooled format, after co-culture with APCs, the activated T cells were sorted based on an activation marker, sequenced to identify the enriched TCRs, and then validated individually. In the arrayed format, individual TCRs were expressed and assayed for tumor reactivity by IFN□ ELISpot. We performed further characterization of tumor-reactive TCRs including cytokine bead array analyses and killing of target APCs to ensure the TCRs are functional. To avoid non-tumor cell toxicity, we used HLA-matched PBMCs to eliminate TCRs that recognize HLA itself or normal proteins.

Results

We identified and validated tumor-reactive T cell clones for multiple patient samples, showing that both autologous tumor and allogeneic cell lines with matching HLA can be used to discover tumor-reactive T cells. Additionally, we use the scRNA-seq data in conjunction with our functional screening data to improve our understanding of the human TIL repertoire. We find that the tumor-reactive TCRs are enriched in the exhausted CD8 cluster but do not solely reside within that cluster. We also find many CD4 tumor-reactive T cells to reside in the regulatory T cell (Treg) population, which we do not advocate including in personalized TCR-T therapy but suggests the tumor microenvironment may have a role in skewing the phenotype of tumor-reactive CD4 to a regulatory T cell.

Conclusions

These results exemplify our approach to affordably identify tumor-reactive TCRs for solid tumor indications. These TCRs can be used to produce highly potent, autologous, polyclonal TCR-T products that have the potential to be the next generation of adoptive cell therapy.

Hybrid T cell receptors with antibody recognition domains improve T cell sensitivity and better recapitulate T cell receptor signaling and synapse formation over CAR T cells

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Adoptive immunotherapy with CAR T cells is effective in hematological malignancies, however tumors expressing low levels of target antigen can escape CAR T cell recognition. Designing receptors that mediate tumor recognition with improved sensitivity over current CARs is required to improve the efficacy of T cell therapies. Work from our lab and others has shown that CARs inefficiently activate canonical TCR signaling pathways, which may underlie their requirement for high levels of antigen compared to T cell receptors. In order to improve CAR T cell sensitivity, while maintaining HLA-independent antigen recognition, novel receptors that fuse antibody variable regions with TCR constant chains were designed, either in a split or single chain format, expressed in T cells and analyzed for signaling, synapse formation and function.

Antibody variable domains specific for CD19 or ROR1 were linked directly to the alpha and beta constant chains of the TCR in a lentiviral vector and expressed in primary human T cells. Base editing was used to delete expression of endogenous TCR chains to prevent mispairing with the introduced chimeric receptor. Signaling in T cells expressing hybrid receptors after antigen engagement was measured using western blot and cytometry and compared to T cells expressing conventional CD19 and ROR1 CARs. Hybrid receptors better engaged canonical TCR signaling pathways, particularly the LAT signalosome, compared to CARs. Hybrid receptors also exhibited improved antigen sensitivity over CARs as measured by in-vitro cytokine production after stimulation with titrated amounts of antigen and in-vivo efficacy in tumor xenograft models in NSG mice. T cell synapses of hybrid receptors and CARs were visualized on a soluble lipid bilayer using TIRF microscopy. T cells expressing hybrid receptors form organized synapses with receptor/cSMAC, ICAM/pSMAC separation after stimulation on a soluble lipid bilayer, unlike CARs which exhibited disorganized synapses. Collectively, these data show that hybrid receptors more closely recapitulate signaling and synapse formation of the TCR than CARs and suggest that using hybrid receptors in adoptive T cell therapies could overcome therapeutic resistance of tumors with low antigen expression.

Identification of patient-specific T cell neoantigens through HLA-unbiased genetic screens

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Accumulating evidence suggests that the efficacy of cancer immunotherapies, such as immune checkpoint blockade or adoptive transfer of tumor-reactive T cells, is to a large extent driven by T cells that recognize cancer neoantigens – T cell antigens that arise as a result of patient-specific tumor mutations. Based on these data, major efforts have been initiated to develop approaches to specifically boost the activity of neoantigen-reactive T cells in individual patients. However, the majority of tumor mutations and their associated neoantigens are unique to individual cancer patients, underscoring the need for technologies that allow the discovery of $CD4^+$ and $CD8^+$ T cell-recognized neoantigens in a truly personalized fashion. Importantly, current methods of T cell antigen identification are generally biased by the use of imperfect prediction algorithms to preselect candidate neoantigens and limited with respect to the breadth of antigen-presenting MHC alleles that is assayed. Thus, experimental tools that enable the comprehensive identification of T cell-recognized tumor antigens on a per-patient basis and across patients' complete MHC haplotypes are required. Here, we present a high-throughput genetic platform for the personalized identification of $CD4^+$ and $CD8^+$ T cell-recognized (neo)antigens. In this method, patient-matched, MHC class I- and class II-proficient B cell lines are engineered to express large libraries of antigen-encoding minigenes, and following co-incubation with patient T cells, the depletion of those B cells that express T cell-recognized epitopes is measured by next-generation sequencing. We leveraged the method to successfully identify $CD4^+$ and $CD8^+$ T cell-recognized neoantigens in patients with diverse solid cancers. Benchmarking against existing tools – including tandem minigene screening and state-of-the-art antigen prediction algorithms – demonstrated enhanced sensitivity of our approach, underlining the value of approaches that allow the unbiased and large-scale identification of patient

neoantigens. Together, these data demonstrate the feasibility of personalized and MHC-agnostic discovery of $CD4^+$ and $CD8^+$ T cell neoantigens from large genetic libraries, and the presented method should facilitate the development of personalized neoantigen-based immunotherapies.

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Immune monitoring in patients with malignant peritoneal mesothelioma treated with adjuvant dendritic cell-based immunotherapy after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy

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Background: Malignant peritoneal mesothelioma (MPM) is a highly aggressive neoplasm with a poor life-expectancy of only 12 months even after (palliative) surgery and/or chemotherapy. Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) have resulted in improved median survival. Nonetheless, even after CRS-HIPEC, recurrence rates remain high. Dendritic cell-based immunotherapy (DCBI) has shown promising results in murine models with peritoneal mesothelioma and in clinical phase I/II studies for patients with pleural mesothelioma, indicating the potential of DCBI after CRS-HIPEC as therapeutic option for MPM.

Methods: Comprehensive immune cell profiling by multicolor flow cytometry was performed on prior to treatment and on treatment (after 1 and 3 vaccinations) peripheral blood samples of 14 MPM treated with DCBI after CRS-HIPEC as part of the ongoing MESOPEC trial (Dutch Trial Registry number NTR7060) in the Erasmus Medical Center, Rotterdam, the Netherlands. Data were analyzed by unsupervised clustering with FlowSOM.

Results: Treatment with DCBI was associated with an increased proliferation of circulating lymphocyte subsets, especially NK cells and CD4+ T-helper cells. In addition, effector memory (Tem) and central memory (Tcm) CD4+ T cells were more abundant after dendritic cell vaccination. Co-stimulatory molecules, including ICOS, HLA-DR and CD28 were upregulated on

CD4+ T-helper and CD8+ T cells after treatment, specifically on memory and proliferating cells and most dominantly on CD4+ T cells. This effect was counterbalanced by significant upregulation of co-inhibitory molecules such as PD-1 and CD39, on both the CD4+ T-helper and CD8+ T cell compartment. Most interestingly, frequencies of terminally differentiated effector memory (Temra) CD8+ T cells and co-expression of ICOS and Ki67 on CD8+ T cells positively correlated with progression-free survival (PFS)

Conclusion: Using comprehensive immune monitoring of peripheral blood of pre-treated MPM patients, dendritic cell vaccination has a diffuse immune modulatory effect on lymphoid cells, mostly on CD4+ T cells. Moreover, CD8+ T cell proliferation and activation seemed to be more pronounced in patients showing a high PFS. This data shed light on immune modulatory effects of dendritic cell vaccination and provide a platform for future combination treatment strategies.

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Improving CAR T therapy by targeting transcription factors driving CAR T cell dysfunction

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CAR T cell immunotherapy has shown remarkable success in a subset of B cell malignancies, but limited clinical efficacy in solid tumors. This therapeutical failure results in part from the acquisition of an exhausted phenotype in CAR T cells due to continuous stimulation by tumor cells expressing the cognate antigen. To gain a deeper understanding of CAR T cell exhaustion induced by chronic antigen exposure (CAE), we developed an *in vitro* model in which mesothelin-redirected CAR T cells (M5CAR) were chronically stimulated with mesothelin-expressing AsPC-1 pancreatic tumor cells for 20-30 days, and we characterized these CAE CAR T cells by gene expression at bulk and single-cell levels. Upon chronic exposure, the M5CAR T cells exhibit a reduced proliferation rate, downregulate the expression of CAR at the cell surface, and showed decreased cytotoxicity. At the transcriptional level, the CAE CAR T cells show an expression signature and an epigenetic landscape consistent with exhaustion that overlaps with datasets of exhausted T cells in chronic infection and cancer. Further, such phenotype was also detected in

post-infused circulating CD19 CAR T cells from DLBCL patients, and in hypofunctional M5CAR T and NY-ESO-1 TCR T cells *in vivo.* By sc-RNAseq, we identified an unbiased dysfunction gene signature encompassing the top 30 genes most highly upregulated upon CAE. Importantly, the M5CAR T cells infiltrating post-infusion ascites collected from pancreatic cancer patients show upregulation of the dysfunctional gene signature identified *in vitro*, suggesting that continuous exposure of CAR T cells to their cognate antigen may be driving CAR dysfunction in the clinic. ID3 and SOX4 were identified as potential drivers of the 30-gene dysfunctional signature. To evaluate the role of each transcription factor in the establishment of the dysfunctional phenotype, we generated ID3KO and SOX4KO M5 CAR T cells. Upon CAE, ID3KO and SOX4KO M5 CAR T cells show superior cytotoxic activity than the WT donor-matched counterparts. Moreover, KO CAR T cells outperform WT CAR T cells *in vivo* and AsPC-1 tumor-bearing xenografts treated with simultaneous ID3 and SOX4 KO M5CAR T cells show durable responses and no recurrence in a mouse model of AsPC-1 tumor relapse. In summary, we have generated an in vitro model that replicates the hallmarks of T cell exhaustion and allowed us to identify a gene signature that characterizes CAR dysregulation and the transcription factors SOX4 and ID3 as key regulators of CAR T cell exhaustion, revealing novel approaches to enhance the efficacy of CAR and TCR T cell therapy in solid tumors.

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In situ tumor arrays reveal early environmental control of cancer immunity

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The immune phenotype of a tumor is a key predictor of its response to immunotherapy. Patients who respond to immune checkpoint blockade generally present with tumors infiltrated by T cells, a phenotype referred to as 'immune-inflamed'. However, not all inflamed tumors respond to therapy, and even lower response rates occur among patients with tumors that lack T cells ('immune-desert') or that spatially exclude T cells to the periphery of the tumor lesion ('immune-excluded'). Despite the importance of these tumor immune phenotypes in patients, little is known about their development, heterogeneity or dynamics due to the technical difficulty of tracking these features in situ.Here, we introduce STAMP (skin tumor array by microporation), a novel preclinical approach that combines in vivo high-throughput time-lapse imaging with next generation sequencing of tumor arrays. Using this approach, we follow the early formation of thousands of clonal tumors to show that the development of a given immune phenotype varies between adjacent tumors; it is not strictly determined by tumor genetics or systemic immunity but rather controlled by local features of the tumor microenvironment. The spatial distribution of T cells, specifically early T cell recruitment by fibroblasts and monocytes into the tumor core, was supportive of T cell cytotoxic activity and tumor rejection. Importantly, tumor immune phenotypes were not static over-time and an early conversion to the 'immune-inflamed' phenotype was predictive of therapy-induced or spontaneous tumor regression. Thus, STAMP captures the dynamic and complex relationships of spatial, cellular and molecular components of tumor rejection and has the potential to translate novel therapeutic concepts into successful clinical strategies.

In vitro expansion of hematopoietic progenitor cells from human pluripotent stem cells

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Hematopoietic progenitor cells from human pluripotent stem cells have the capacity to generate T-cells, B-cells, NK-cells, and macrophages in vitro. Pluripotent stem cells from patient's

skin cells are an ideal source of autologous adoptive cell therapy. The large-scale production of hematopoietic progenitor cells holds promise in the adoptive immunotherapy of cancer. We hypothesize that the modulation of hematopoietic signaling pathways expands hematopoietic progenitor cells in vitro. We previously established an organoid-based robust production of hematopoietic progenitor cells from human pluripotent stem cells (Chao et al., 2022. Biorxiv). We propose the mid-throughput chemical screening platform of hematopoietic progenitor cells in a 96-well format. We read out i) proliferation by cell numbers, ii) stemness by surrogate phenotypic markers CD34, CD43, and CD45. Our pipeline tests signal modulators that increase human cord-blood hematopoietic stem and progenitor cells such as SR1, UM171, and polyvinyl alcohol, and kinases whose mutations are major drivers in myeloproliferative neoplasms such as JAK and PI3K. Here we present our results of the screening. In vitro expansion of hematopoietic progenitor cells from human pluripotent stem cells will boost adoptive immunotherapy.

In Vitro Modeling of CD8 T Cell Exhaustion Uncovers Novel Transcriptional Regulator Bhlhe40

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Identifying novel molecular mechanisms of exhausted CD8 T cells (T_{ex}) is a key goal of improving immunotherapy of cancer and other diseases. However, high-throughput interrogation of *in vivo* T_{ex} can be costly and inefficient. *In vitro* models of T_{ex} are easily customizable and quickly generate high cellular yield, offering an opportunity to perform CRISPR screening and other high-throughput assays. We established an *in vitro* model of chronic stimulation and benchmarked key phenotypic, functional, transcriptional, and epigenetic features against *in vivo* T_{ex} generated during chronic LCMV infection. We then leveraged this model to perform pooled CRISPR screening to identify novel transcriptional regulators of T cell exhaustion. This approach identified several transcription factors, including Bhlhe40. *In vitro* and *in vivo* validation defined a role for Bhlhe40 in regulating a key differentiation checkpoint between progenitor and intermediate subsets of T_{ex}. Thus, by developing and benchmarking an *in vitro* model of T_{ex}, we demonstrate the utility of mechanistically annotated *in vitro* models of T_{ex}, in combination with high-throughput approaches, as a discovery pipeline to uncover novel T_{ex} biology with therapeutic relevance for cancer immunology.

Insufficient activation of CAR-T cells in tumor-draining lymph nodes correlates with their poor persistence relative to TCR-T cells in solid tumors

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Adoptive cell therapy (ACT) with engineered T cells has exhibited remarkable success against hematological malignancies, but this success has yet to extend to solid tumors. The rapid onset of T cell exhaustion is one major barrier that limits the efficacy of engineered T cells in solid tumors by promoting their dysfunction and poor persistence. Antigen-presenting cells (APCs) regulate T cell exhaustion and coordinate productive T cell responses to solid tumors. APCs maintain progenitor exhausted T cells (Tpex) in tumor-draining lymph nodes (tdLNs) that replenish T cells as they exhaust in tumors. APCs also promote the survival of effector-like exhausted T cells that differentiate from Tpex and mediate tumor killing. Thus, APCs are crucial for maintaining adequate numbers of functional T cells in tumors.

T cell exhaustion and its regulation by APCs has been well-described for endogenous T cells, **but it is not known whether exhaustion of engineered ACTs is regulated by APCs in the same way**. Engineered ACTs are pre-activated *in vitro* before infusion, which may enable them to bypass dependency on APCs *in vivo*. Further, there are two primary types of engineered ACTs: TCR-T and CAR-T cells. TCR-T cells recognize tumor-associated antigens presented on MHC by tumor cells and APCs. By contrast, CAR-T cells are MHC-independent and only recognize intact surface antigens on tumor cells. We hypothesize that CAR-T cells bypass activation by APCs in tdLNs and that this impairs their persistence and function relative to TCR-T cells in tumors.

To test this, we used the clinically relevant $Kras^{LSL\text{-}G12D/+;p53fl/fl}$ (KP) mouse model for lung adenocarcinoma. We engineered KP tumors to co-express the CAR target ROR1 and the TCR target ovalbumin (Ova) to enable head-to-head comparison of tumor-specific CAR-T and TCR-T cells. ROR1-specific CAR-T and Ova-specific OT-I TCR-T cells were activated and cultured identically prior to co-transfer into tumor-bearing $KP^{ROR1/Ova}$ mice. OT-Is preferentially accumulated and exhibited features of activation in tdLNs relative to non-tumor draining lymph nodes (ndLNs), including increased proliferation and adoption of a $PD\text{-}1^{+}Tcf1^{+}$ exhausted Tpex phenotype. By contrast, CAR-T cells showed similarly low accumulation and proliferation in tdLNs and ndLNs alike and maintained a $PD\text{-}1^{-}Tcf1^{+}$ phenotype similar to their pre-infusion phenotype, suggesting that CAR-T cells fail to be activated in the tdLN. Consistent with their increased accumulation in tdLNs, OT-Is drastically outnumbered ROR1 CAR-T cells in tumors,

suggesting a correlation between tdLN accumulation and persistence in tumors. Indeed, blocking lymph node egress with the S1PR1 modulator FTY720 resulted in a significant decline of both ROR1 CAR-T and OT-I TCR-T cell numbers in tumors, indicating that LNs play a functional role in maintaining the persistence of engineered T cells within tumors.

Together, our data show that, compared with OT-I TCR-T cells, ROR1 CAR-T cells are not activated and do not accumulate in tdLNs and that this correlates with their poor accumulation and persistence in tumors. Our work suggests that CAR-T cells bypass activation by APCs in tdLNs due to their MHC-independent design, and that this may represent a therapeutic vulnerability contributing to poor persistence in tumors.

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Integrated immuno-genomic analyses of high-grade serous ovarian cancer reveal vulnerability to combination immunotherapy

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Cancer immunotherapy has shown limited therapeutic efficacy in high-grade serous ovarian cancer (HGSOC). To overcome these limitations, a number of clinical trials combining immunotherapies with poly-ADP-ribose polymerase (PARP) inhibitors are currently underway. Hence there is an urgent need to identify predictive biomarkers. We performed multi-omics data analyses of the TCGA HGSOC cohort (n=226) and based on genomic instability (mutation of homologous recombination repair (HR) genes, HR deficiency score, mutational signature 3, and BRCA1/2 promoter methylation) defined BRCAness. Using random forest we could identify a 24 gene expression signature to determine the BRCAness status of a tumor (10 fold CV AUC 0.89±0.11). Immune characteristics including tumor infiltrated immune cells, immunophenoscore, molecular subtype, enrichment of immune related signatures such as T cell inflammation and CD8 exhaustion were analyzed based on gene expression data. Tumor-immune phenotype was obtained from a classifier based on digital pathology of CD8 T-cell distribution. Furthermore, HLA types, tumor mutational burden, neoantigens, and immunoediting history were estimated. Whereas TMB and neoantigen load was different between BRCAness and non-BRCAness samples ($p<0.001$) other immune parameters such as T cell inflammation showed only a moderate positive association with BRCAness (rho=0.16,

p=0.018). However, we could show by in vitro data (RNA seq, IF) in a BRCA1-/- cell line (UWB1.289) treated with the PARPi Olaparib an interferon type I response and cGAS-STING pathway activation indicating a potential activation of the (innate) immune system. In order to identify potential responders to combination immunotherapy we divided the BRCAness tumors – which indicate a potential PARPi response – into a group with both an infiltrated tumor-immune phenotype and immune reactive molecular subtype (IMR) and a group including all other combinations. Although CD8+ T cells were more abundant in the immune group (p<0.001) we could not observe a different effect on overall survival between these groups (HR=0.81, 95%-CI: 0.42-1.60, p=0.55). This could be due to the observed higher CD8+ Tcell exhaustion (p<0.001), and increased immune suppressive environment including Tregs (p<0.001), MDSCs (p<0.001), and M2 macrophages (p<0.001). Although this environment could be adverse for immunotherapy a recent clinical study indicate also for some responder a T cell exhaustion and macrophage infiltration. Therefore, we propose a score to predict response to combination therapy that indicates BRCAness with a favorable balance between activated and suppressive immune environment. Results were validated on tumor samples of patients on a HGSOC cohort (n=60) from the Medical University of Innsbruck using RNA sequencing and immunohistochemistry analyses. In summary, we propose a novel diagnostic algorithm for PARPi immune checkpoint combination therapy and provide a software (R package) for comprehensive RNA sequencing based characterization of HGSOC samples.
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Integration of multi-omic data into immune modules predictive of response to checkpoint blockade in cutaneous T cell lymphoma

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Immunotherapy has greatly increased overall survival across a broad range of malignancies, yet many individuals still fail to mount an effective anti-tumor response. Accurate biomarkers are needed to identify patients who are good candidates for immunotherapy and additional therapeutic approaches are needed to treat patients refractory to existing therapies. To discover important drivers of successful anti-tumor immunity, researchers apply multiple, high-dimensional assays to primary samples from clinical trials, but integrating and analyzing these

large, disparate datasets from relatively small cohorts in a robust and interpretable manner remains a substantial challenge. Here, we propose a new analytic framework integrating features from multiple omics technologies into interpretable immune modules associated with therapeutic response.

We applied a systems immunology approach to investigate the endogenous and neoplastic immune response in cutaneous T cell lymphoma patients undergoing either anti-PD-1 monotherapy or interferon gamma 1-b and anti-PD-1 combination therapy. To characterize the systemic, local, and cellular response to these therapies, we interrogated longitudinal peripheral blood samples by single-cell mass cytometry, TCR immune repertoire sequencing, and serum proteomics, and we employed high-dimensional proteomic imaging (CODEX) and bulk transcriptomics on longitudinal tumor biopsy samples. To identify correlates of response, we organized our large multi-omic feature set into data-intrinsic immune modules and quantified module importance using a nonlinear machine learning classification method. This approach not only identifies features associated with therapeutic response but can also generate new hypotheses about co-regulation and dependencies, providing new mechanistic insights into checkpoint blockade biology as well as putative biomarkers for predicting overall response.

Investigating the expression and therapeutic potential of the inhibitory immune checkpoint VISTA in hepatocellular carcinoma

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Introduction:

Immune checkpoint blockade (ICB) has shown encouraging clinical activity for the treatment of advanced hepatocellular carcinoma (HCC), with combination therapies achieving objective response rates of up to 30% and extending survival. Still, there is an unmet need to identify

novel immuno-oncology drug targets and combinations, and elucidate the molecules and pathways responsible for intrinsic and acquired resistance in HCC, to further improve outcomes.

VISTA (V-domain Ig-containing Suppressor of T cell Activation) is an immunosuppressive checkpoint expressed predominantly on haematopoietic cells, particularly myeloid cells. The VISTA pathway has been highlighted as a possible resistance mechanism to ICB in prostate cancer and melanoma, and VISTA blockade with anti-VISTA antibodies has been shown to inhibit tumour growth in multiple non-HCC cancer models. However, VISTA is understudied in HCC.

Hypothesis:

We hypothesise that VISTA plays key roles in immunosuppression and potentially intrinsic or acquired resistance to immunotherapy in HCC, and might thus serve as a novel drug target for HCC treatment, as the HCC microenvironment is rich in diverse myeloid cell types that inhibit optimal anti-tumour immunity. We thus investigated the expression and therapeutic potential of VISTA in HCC.

Methods and Results:

We assessed VISTA expression in HCC patient samples through single-cell RNA-seq (n=10), multiplexed immunohistochemistry (n=45), and flow cytometry analyses of surgically resected tumour and non-tumour samples. We found a high frequency of $VISTA^{+}$ granulocytes and myeloid cells in both tumour and non-tumour tissue, with little to no VISTA expression on T, B and NK cells.

We evaluated the therapeutic efficacy of a commercially available anti-mouse VISTA antagonist antibody in an immunocompetent syngeneic mouse model of HCC. We measured tumour growth, and analysed the tumours, spleen, and tumour-draining lymph nodes by high-parameter flow cytometry. We found significant tumour growth inhibition with anti-VISTA monotherapy in our HCC model (n=10 per treatment group). This was accompanied by decreased $Ly6G^{+}$ neutrophils, $CD4^{+}Foxp3^{-}$ T cells, $CD4^{+}Foxp3^{+}$ Tregs, and $Ly6C^{hi}$ macrophages, and increased $Ly6C^{lo}$ macrophages, in the tumour tissue.

Conclusions:

In our study, VISTA is highly expressed in myeloid cells in Asian, largely Hepatitis B-driven HCC. Moreover, VISTA blockade exerts an anti-tumour effect even as monotherapy, inhibiting tumour growth and modulating the immune microenvironment. These results provide a rationale for pursuing further studies into VISTA targeting and function in HCC, and suggest that anti-VISTA therapeutics could potentially be part of the HCC clinical therapeutic armamentarium.

Funding:

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JAK Inhibition with Anti-PD1 Improves CD8 T Cell Differentiation and Response in Lung Cancer

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NSCLC (Non Small Cell Lung Cancer) remains the leading cause of cancer mortality despite the success of immune checkpoint blockade (ICB) as first-line therapy. Although patients can be stratified into therapeutic response groups using cancer associated features like low tumor mutational burden or PDL1 expression, cancers can acquire resistance mechanisms to ICB which subverts anti-tumor immunity. The induction and persistence of inflammation including type-one interferon (IFN-I) is one mechanism associated with immunosuppression. We show that delayed administration of itacitinib ,a JAK1 inhibitor, after αPD1 improves immune function and anti-tumor responses in mice. The effects of itacitinib and αPD1 can be phenocopied with αIFNAR1 and αPD1 blockade suggesting a shared mechanism in improving responses to ICB. These results prompted the initiation of a phase-2 clinical trial for metastatic NSCLC with tumor PDL1≥50% (NCT03425006). Treat naïve patients received 2 cycles of pembrolizumab (200mg every 21 days), followed by 2 cycles of pembrolizumab and 6 weeks of itacitinib (200mg daily po), followed by pembrolizumab alone until disease progression. In our study, the best overall response rate (BOR) was 67% with median progression-free survival (PFS) of 27.6 months—substantially higher than comparable randomized studies (NCT02142738) which reported BOR

of 44% and median PFS of 6.5 to 10.3 months. We found that patients with low IFN-I inflammation responded to pembrolizumab; whereas patients with elevated IFN-I inflammation were initially unresponsive to αPD1, but turned αPD1-responsive after itacitinib. Itacitinib promoted CD8 T cell plasticity and improved therapeutic responses of exhausted and effector-memory clonotypes. In contrast, patients with persistent IFN-I signaling were refractory to itacitinib and showed progressive CD8 T cell terminal differentiation and progressive disease. Thus, JAK1 inhibition may improve αPD1 efficacy by pivoting CD8 T cell differentiation dynamics

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Lactic acid uptake through MCT11 enforces dysfunction in terminally exhausted T cells

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Upon infiltration into tumors, $CD8^+$ T cells experiencing persistent antigen stimulation progressively differentiate into a state of dysfunction, known as exhaustion. Exhausted T cells are characterized by the upregulation of co-inhibitory molecules and reduced effector cytokine production. Additionally, exhausted T cells exist in a state of metabolic dysfunction in the tumor microenvironment (TME), due to disrupted mitochondrial biogenesis, hypoxia and a lack of metabolites. Highly glycolytic tumor and stromal cells outcompete T cells for glucose, and secrete lactic acid into the TME, acidifying the extracellular space. Recent studies have shown that lactate can be incorporated into the TCA cycle by $CD8^+$ T cells and that it can be utilized in the TME as a fuel source by regulatory T cells and macrophages. We hypothesized that $CD8^+$ tumor-infiltrating lymphocytes (TIL) may take also up lactate as an alternative carbon source to meet their metabolic demands in the TME. RNA sequencing and flow cytometry data from $CD8^+$ T cell in the TME revealed MCT11 (encoded by *Slc16a11*), a monocarboxylate transporter only recently discovered and described, to be highly and uniquely expressed in terminally exhausted T cells (Tex). As lactic acid is a tumor abundant monocarboxylate, we asked whether MCT11 supports lactate uptake into Tex cells. Culturing FACS sorted TeX cells in 14[C]-lactate revealed

that these cells had increased capacity of oxidizing lactate than draining lymph node $CD8^+$ T cells and progenitor exhausted T cells (Pex). Genetic and antibody blockade of MCT11 resulted in reduced 14[C]-lactate oxidation by Tex cells, but it remained unclear if lactic acid promoted or inhibited effector function. Overexpression of MCT11 in OT-I T cells adoptively transferred into B16-OVA bearing mice resulted in accelerated exhaustion: increased co-inhibitory marker expression and decreased TNFα and IFNγ production. Conversely, tumor bearing mice with a conditional knockout of MCT11 in T cells (*Slc16a11*$^{f/f}$xCD4^{cre}) had an increased total CD8+ TIL in the tumor, increased production of TNFα and IFNγ production by $CD8^+$ TIL, and decreased tumor burden in mice. As MCT11's uptake function was blocked with an antibody, we also used the antibody therapeutically in tumor bearing mice, revealing that single-agent MCT11 antibody therapy led to complete response (CR) in 40% of mice bearing MEER tumors. Additionally, in the MC38 tumor model, combination therapy of anti-MCT11 with anti-PD1 led to 80% CR, while anti-PD1 therapy alone led to tumor clearance in 40% of mice. Our data support a model where exhausted CD8+ T cells upregulate MCT11, which renders them sensitive to toxic lactic acid present at high levels in the tumor microenvironment. Our data suggest MCT11 could be deleted on therapeutic T cells or blocked on endogenous T cells to render exhausted T cells impervious to lactic acid such they can mediate tumor eradication.

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Late CTLA-4 Ig treatment improves antitumor efficacy of immunotherapy

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Combining immune checkpoint therapies such as anti-CTLA-4 and anti-PD-1 increases antitumor response and overall survival rate relative to single treatments. However, it also increases the frequency and severity of immune-related adverse events (irAEs), such as cardiotoxicity. Our previous results showed that CTLA-4 Ig (abatacept), an inhibitor of T cell costimulation through CD28, can reverse those irAEs in patients with cancer, however, concern remains that it could blunt the antitumor response of immune checkpoint therapy (ICT). In the B16F10 melanoma model, we injected CTLA-4 Ig antibody into mice: 1) along with anti-CTLA-4, anti-PD-1, or combination (early time point) or 2) after ICT treatment is completed (late time point). We demonstrated that CTLA-4 Ig at the early time point compromised the antitumor efficacy of the ICT. Unexpectedly, the antitumor efficacy of the ICT was improved if mice were treated with CTLA-4 Ig at the late time point. The frequency and function of the terminally differentiated CD8 effector T-cells were not affected. In contrast, ICOS+ Foxp3+ Tregs was depleted by CTLA-4 Ig

(late). Collectively, we show that CTLA-4 Ig treatment has differential roles throughout the time course of ICT treatments, and this work provides a mechanism by which CTLA-4 Ig (late) treatment competes with CD28 on Tregs which constantly require costimulation from CD80 and CD86, thus depleting Tregs in the tumor microenvironment and improving the antitumor efficacy of ICT treatments.

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Longitudinal profiling of blood monocytes during human lung cancer progression reveals their pro-tumor phenotype and identifies immuno-metabolic regulation

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The dynamic role of monocytes/macrophages across cancer progression, treatment and relapse response is not well-understood. To this end, we studied Non-Small Cell Lung Cancer (NSCLC) patients longitudinally at baseline (treatment naïve), during targeted therapy and at relapse. Evolution of immune landscape across these phases was studied using flow cytometric phenotyping of blood immune subsets, plasma cytokine profiling and focused transcriptomics on blood monocytes. Monocyte transcriptome showed a distinct modulation in their gene expression profile at baseline versus during treatment. At baseline, monocytes showed upregulation of genes associated with cancer-related inflammation, activation receptors and amino acid/lipid metabolism. Validation using baseline monocytes and in vitro tumor-conditioned monocytes demonstrated that the differentially regulated immuno-metabolic genes and their metabolite(s) was responsible for inducing a pro-tumor phenotype in monocytes. These data suggest the potential of immunotherapeutic targeting of monocytes and/or their immuno-metabolic circuits to re-program them to support an anti-tumor response in NSCLC.

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Manipulating T cells for Adoptive Cell Transfer: Balancing improved effector function versus increased exhaustion

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Adoptive cell therapy (ACT) is a promising strategy for treating cancer, but the optimal cell phenotype for ACT is still being explored. One major barrier to successful ACT is T cell exhaustion, induced by chronic TCR stimulation in the tumor microenvironment. Deletion of the phosphotyrosine phosphatase, non-receptor type 22 (PTPN22) enhances response to antigen, and we have previously reported that ACT of PTPN22$^{-/-}$ naïve or memory CD8+ T cells improves in vivo tumor control. However, the effect of loss of PTPN22 in effector CTL is less defined, in particular the impact on CTL exhaustion in response to tumors. Here, we show that deletion of PTPN22 in CTL decreases their ability to control tumor growth while increasing their rate of exhaustion in response to persistent antigen exposure. Adoptively transferred PTPN22$^{-/-}$ OT-1 CTL controlled weak SIITFEKL (T4) ovalbumin peptide expressing MC38 tumor growth less effectively than WT OT-1 CTL, despite having improved cytokine and cytotoxic effector function at the time of transfer. In response to repeated re-stimulation with antigen, PTPN22$^{-/-}$ CTL were characterized by more rapid loss of these effector functions than WT CTL. This coincided with earlier and higher expression of inhibitory receptors (IRs), and the anti-tumor response of PTPN22$^{-/-}$ CTL was improved following PD-1 blockade in vivo, suggesting the PTPN22$^{-/-}$ cells were exhausting more readily. PTPN22$^{-/-}$ cells expressed very high levels of the IR and terminal exhaustion marker TIM-3 in particular. However, knock out or mAb blockade of TIM-3 in PTPN22$^{-/-}$ CTL did not improve but instead further impaired tumor control following ACT, suggesting TIM-3 signaling itself does not drive the diminished function seen in PTPN22$^{-/-}$ CTL. Using TIM-3 expression as a readout of the underlying signaling in PTPN22$^{-/-}$ CTL, we found an important role for IL-2 signaling to the transcription factor NFIL3, with a further boost from subsequent TCR re-stimulation. Our findings demonstrate that although deletion of PTPN22 in naïve or memory CD8+ T cells improves tumor responses, in CTL the loss of restraint on TCR signaling may conversely drive the cells more rapidly to exhaust in the presence of continuous antigen exposure, such as in the tumor microenvironment, and that this is not reversed by targeting TIM-3. This highlights the need for careful choice of cell for ACT, in order to balance short term augmented effector function with longer term protection.

MicroRNA manipulation of Macrophage Polarisation status

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Tumour Associated Macrophages (TAM) that are CD163 positive are associated with poor response to immunochemotherapy in patients with Diffuse Large B Cell Lymphoma (DLBCL) and make up a significant proportion of the tumour microenvironment (TME). Targeting these TAMs to reverse immunosuppression is one potential approach for improving treatment response rates.

A change in microRNA (miR) expression has the potential to modify hundreds of genes by interacting with multiple mRNA targets, thereby altering the wider transcriptional profile and activity of cells. The manipulation of miRs that control macrophage behaviour may prove beneficial in switching the global transcriptomic profiles of immunosuppressive 'M2-like' macrophages to more activated, anti-tumour states resulting in the reversal of immune resistance and enhancement of monoclonal antibody (mAb) therapy.

Methods

MiRNAs differentially expressed in DLBCL and associated with poor response were identified using bioinformatics analysis of DLBCL datasets. Peripheral blood mononuclear cells (PBMCs) were obtained from normal blood donors and polarised to an 'M2-like' phenotype using IL4 and IL13 or an 'M1-like' phenotype using LPS and IFNγ following 7 days of culture with MCSF. MiRNA expression was measured using RT-qPCR and presented as fold change relative to a non-polarised control (M0).

To assess impact on antibody dependent cellular phagocytosis (ADCP), macrophages were generated as above, polarised to an 'M2-like' phenotype and transfected with anti-miRs (thermofisher) 48 hours prior to co-incubation with target cells. Human Chronic Lymphocytic Leukaemia (CLL) cells from patient samples were labelled with CSFE and used as target cells with Rituximab as the ADCP-inducing mAb and Herceptin as an isotype control. Macrophages, identified by their Fc gamma receptor IIIA (FcγRIIIA) positivity were used to determine the percentage of double positive cells following co-incubation using flow cytometry and analysed using FCS express software. The phagocytic index (PI) was calculated for each condition by subtracting the average percentage of double positive cells in the Herceptin group from the percentage double positive cells in the Rituximab group and dividing this by the M0 value to calculate a ratio.

Results

There was significant upregulation of miR 142-5p in M2-like macrophages 48 hours following polarisation compared to M1-like macrophages and non-polarised M0 controls. Inhibition of miR 142-5p expression in M2-like macrophages led to a significant increase in FcγRIIIA surface expression with a subsequent trend towards enhanced phagocytosis of CLL cells opsonised with Rituximab in vitro when compared with M2-like controls.

Conclusions

Transfection of anti-miR 142-5p can repolarise macrophages to a more activated anti-tumour phenotype by recalibrating FcγR profiles leading to enhanced mAb efficacy.

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microRNA-29a promotes T cell responses to anti-PD-1 therapy

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Cancer causes CD8 T cell exhaustion, a differentiation state characterized by the inability of CD8 T cells to provide immunological protection. Improving the function of exhausted T cells (T_{EX}) by antagonizing checkpoint inhibitors is a potent immunotherapeutic strategy. However, a large number of patients do not benefit from immunotherapy or do not exhibit long-term protection, creating a need for improved immunotherapeutic strategies. Failure to respond to immunotherapy is attributed to the epigenetic stability of T_{EX} and inability of checkpoint inhibitors to reverse T_{EX} differentiation. Transcriptional and epigenetic analyses of T_{EX} identified cellular and molecular pathways associated with exhaustion. Most, if not all, of these exhaustion-associated pathways are regulated by microRNAs (miRs); therefore, miRs represent novel ideal targets for immunotherapy. We recently demonstrated that miR-29a redirects T_{EX} differentiation into memory (T_{MEM})-like differentiation; therefore, presents an attractive target for novel immunotherapeutic strategies. Using retroviral overexpression of miR-29a as well as miR-29a deficient T cells, we demonstrate that miR-29a attenuates exhaustion, enhances long-term persistence of CD8 T cell in mouse models of chronic infection and tumors and promotes protective anti-tumor CD8 T cell responses. Importantly, miR-29a synergizes with checkpoint blockade therapy to promote a T_{MEM}-like phenotype and enhance long-term protective CD8 T

cell responses. Mechanistically, miR-29a altered the differentiation of the progenitor exhausted CD8 T cell subset expressing the transcription factor TCF-1, as TCF-1+ progenitor T_{EX} overexpressing miR-29a responded to anti-PD-1 blockade more effectively compared to control TCF-1+ T_{EX}. Therefore, we propose that miR-29a is a novel regulator of T_{EX} differentiation that can predict and enhance responsiveness to checkpoint blockade therapy.

Molecular determinants of Acute Myeloid Leukemia targeting by Delta One T cells

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Cytotoxic gd T cells constitute an attractive effector population for cancer immunotherapy. We have recently developed an adoptive cell therapy based on the $Vd1^+$ subset of gd T cells, so-called Delta One T (DOT) cells, and showed its therapeutic potential in pre-clinical models of Acute Myeloid Leukemia (AML). However, the mode-of-action of DOT cells against AML remains unclear, especially as our previous data suggested a TCR-independent targeting mechanism. Here we investigated the role of NK cell receptors (highly expressed on DOT cells), and their tumor-associated ligands, in AML targeting by DOT cells. Screening of mRNA and protein expression levels for candidate NK receptor ligands showed that the DNAM-1 ligands, PVR and Nectin-2, were highly and robustly expressed in AML cell lines and primary samples. By employing the CRISPR/ Cas9 gene editing methodology, we were able to pinpoint a major role for PVR in AML targeting, whereas the impact of Nectin-2 was negligible. Furthermore, the ablation of the NKp30 ligand, B7-H6, had an additive effect in PVR-deficient AML cells, leading to a marked reduction of DOT-cell cytotoxicity. This was also reflected in the substantial impairment in immunological synapse formation between DOT cells and AML cells lacking PVR and B7-H6. Importantly, blockade of DNAM-1, NKp30 and their ligands also reduced the ability of DOT cells to lyse primary samples obtained from AML patients. While the clinical significance of these findings remains to be established, our results shed new light into the mode-of-action of DOT cells and suggest that PVR and B7-H6 expression may be important biomarker(s) to assess in AML blasts when evaluating their therapeutic efficacy.

Molecular, Metabolic and Functional CD4 T cell Paralysis through the Interplay of Multiple Immune Checkpoints Impedes Tumor Control

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CD4 T cells are central effectors of anti-cancer immunity and immunotherapy, yet the regulation of CD4 tumor-specific T (T_{TS}) cells during cancer progression is unclear. In this study, we identify that a rapid and sustained multiparameter paralysis of CD4 T_{TS} cells facilitates tumor immune evasion and growth. We demonstrate that CD4 T_{TS} cells are initially primed and start to divide following tumor initiation. However, proliferation of CD4 T_{TS} cells is rapidly suspended, stunting their differentiation, redirecting metabolic circuits, and abrogating accumulation in the tumor. The “paralyzed” state is distinct to tumor-antigen specific CD4 T cells and is different from the previously described anergy or T cell exhaustion. Mechanistically, the paralyzed state is imposed and then actively sustained by a functional interplay of regulatory T cells (Treg) and CTLA4. These signals together prohibit CD4 accumulation in the tumor, although a fraction of the paralyzed CD4 T_{TS} cells are converted to Treg themselves over time, providing a mechanistic explanation for the clinical observation of shared TCR clonotypes among different CD4 Th subsets. In contrast, CD8 T_{TS} cells did not exhibit the proliferative paralysis or tumor homing inhibition indicating a distinct set of mechanisms set up by the tumor to limit CD4 T cells. The paralysis is actively maintained throughout cancer progression and CD4 T_{TS} cells rapidly resume proliferation, functional differentiation and tumor infiltration when the Treg- and CTLA4-mediated suppressive constraints are alleviated. Repairing their paralysis induces rapid and long-term tumor control, demonstrating the fundamental necessity of rapidly crippling CD4 T_{TS} cells for tumor progression and their potential importance as therapeutic targets.

mregDC, Tfh-like CD4+ T cell niches enable local reinvigoration of tumor-reactive CD8+ T cells following PD-1 blockade in human

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While enrichment of T cells in tumors is a well-established predictor of response to immunotherapy, many T-cell-rich tumors fail to respond to immune checkpoint blockade (ICB). Taking advantage of the largest trial of neoadjuvant PD-1 blockade in hepatocellular carcinoma (HCC) to date, we searched for correlates of pathological response within T-cell-rich tumor lesions. We profiled nearly 1 million immune cells from paired tumor and adjacent tissues, and noted significant expansion of $CH25H^{+}CXCL13^{+}IL\text{-}21^{+}CD4^{+}$ cells with T follicular helper (Tfh) features and $GranzymeK^{+}PD\text{-}1^{+}CD8^{+}$ cells with effector features in responders compared to T cell rich non-responders. Using TCR sequencing and Basescope TCR imaging, we show that T cells expand and differentiate locally from pre-existing $CD4^{+}$ and $CD8^{+}$ clones along two trajectories in responders and non-responders, prompting our search for spatial determinants of response. We found that tumors from responders were enriched in spatially organized and physically interacting cellular triads that included mregDCs, a DC state triggered by capture of tumor antigens, $CD4^{+}$ Tfh-like T cells and $CD8^{+}$ T cells with features of progenitors. Receptor-ligand analysis revealed unique cellular interactions within these triads that may promote progenitor $CD8^{+}$ T cell differentiation into effector cells upon PD-1 blockade. These results suggest that discrete cellular niches that include mregDCs and Tfh-like $CD4^{+}$ T cells may control

the reactivation of pre-existing tumor-specific progenitor CD8$^+$ T cell clones into effective anti-tumor T cells, and that promoting these triads could significantly enhance tumor response to ICB.

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Multi-dimensional investigation of tertiary lymphoid aggregates in immunotherapy-treated pancreatic ductal adenocarcinoma

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Tertiary lymphoid aggregates (TLA) are immune cells spatially organized into ectopic lymphoid centers that can assemble germinal centers (GC). These structures have been identified in solid tumors in various tissue types and have been associated with patient outcomes such as overall survival and responses to immunotherapy. Pancreatic ductal adenocarcinoma (PDAC) generally presents with minimal immune infiltration and TLA formation which contributes to a particularly low five-year survival rate and limited beneficial response to single-agent immunotherapy. Using a granulocyte-macrophage colony-stimulating factor secreting allogeneic vaccine (GVAX) we previously demonstrated the induction of TLA in PDAC patients two weeks post vaccine treatment. Immunohistochemistry studies of vaccine-induced PDAC TLA revealed cellular compositions indicative of an adaptive immunity response, such as B and Cytotoxic T cells. However, the induction of these TLA still did not result in significant clinical benefit. Using spatial proteomics and transcriptomics platforms, we characterize the molecular landscape of vaccine-induced TLA in PDAC tumors as well as lymph nodes with metastatic PDAC in patients treated either with GVAX or GVAX and Cyclophosphamide. To identify genome-wide spatially variable features that distinctly identify immune related patterns in the PDAC TLA transcriptional

landscape, we applied nonnegative matrix factorization using CoGAPS. This unsupervised approach identified distinct coregulated gene expression patterns. We computed corresponding significant genes using patternMarkers to define the molecular identity of these patterns. Additionally, we projected patterns into a PDAC single cell RNA sequencing atlas to deconvolute the cellular composition of these patterns. We extracted a gene expression pattern that specifically identified cellular communities in TLA and GC, as well as immunoglobulin enriched patterns indicative of plasma cell infiltration which were spatially restricted near neoplastic regions. Since peritumoral and intratumoral TLA have different degrees of prognostic value in cancer, we computed TLA distances to neoplastic regions and identified molecular distinctions in TLA as a function of distance to PDAC. Notably, we found that proximity of TLA to neoplastic lesions significantly correlated with increased B cell activation. Our work provides comprehensive spatial molecular profiling of the PDAC TME and navigates the spatial heterogeneity of the immune landscape in a typically non-immunogenic tumor revealing new avenues for immunotherapy exploration.

Multi-omic machine learning approach to predict cancer immunotherapy response in melanoma

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Introduction

Cancer immunotherapy has shown great promise in improving patient survival for a variety of cancer types, including melanoma and lung cancer. However, many patients eventually develop resistance or undergo severe toxicity to immune checkpoint inhibitors (ICIs)[1]. Hence, there is a need to predict the clinical and molecular factors of responders and non-responders to ICIs. Parallel to the development of ICIs, next generation sequencing technologies and bioinformatics tools enabled the characterization of the tumour micro-environment and the complex immune interactions. Earlier studies used whole genome, exome and RNA sequencing in melanoma identified key factors such as tumour mutation burden and cytolytic activity are associated with responders to ICIs[2-4]. However, these factors may depend on other numerous confounding clinical and molecular features. On the other hand, machine learning methods are capable of

integrating multiple types of omic data and help to decipher the nature of complex tumour immune interactions as well as predict ICI response from multi-omics studies.

Materials and methods

In our current study, we utilized our whole genome (the Newell et al, 2022 dataset[5]), publicly available exome and RNA datasets to train a multi-omic machine learning classification model. The multi-omic ensemble model comprised of (i) a logistic regression (ii) a random forest and (iii) a support vector machine, and was trained using a combination of clinical, DNA and RNA sequencing features from the Newell et al, 2022[5] dataset. Multiple validation datasets[2,4-7] including Rozeman et al, 2021[6] were used to evaluate the trained model's performance, as shown by area under curve (AUC) and receiver operating characteristic (ROC) statistics calculated from ICI response prediction. Feature importance analysis was also conducted on the trained model to identify key features influencing ICI response.

Results and discussion

We report an integrated multi-omics approach that could be used to predict responses to ICIs in melanoma. Over 46 hyperparameters were evaluated from clinical, DNA and RNA sequencing features. The ensemble model was trained independently and integrated on all features, evaluated response prediction and validated in a test dataset. Here we observed, a key few DNA-based hyperparameters including TMB and neoantigen load, RNA based deconvolution hyperparameters were associated in responders. We will present the findings from our best predictive model and describe the key clinical and molecular features of responders to immunotherapy.

Conclusion

State of art integrated multi-omics on ensemble machine learning approach is able to predict ICIs responses in melanoma. With more comprehensive clinical and molecular datasets and prospective validation, we believe our approach could inform clinical practice.

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Multispecific hybrid T cell receptors for sensitive targeting of multiple myeloma

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CAR T cells targeting MM associated antigens BCMA, GPRC5D, CD229 and SLAMF-7 are promising therapeutic options for the treatment of multiple myeloma. But despite good initial response rates, long term remissions remain infrequent and 50-60% of treated patients will relapse within 1 year after CAR T cell infusion. Heterogeneity of target antigen expression on the tumor can lead to selection of tumor clones with low levels of antigen and/or loss following immune pressure with CAR therapy. Conventional CARs that encode CD3z and a costimulatory domain require high levels of antigen for efficient T cell activation and tumor elimination. By contrast, T cell receptors (TCRs) are 10-100 times more sensitive than CARs as they engage a more complex and diversified signaling machinery following antigen recognition. To achieve greater antigen sensitivity, we and others designed synthetic hybrid T cell receptors by fusing antibody-based recognition domains to TCR alpha and beta constant chains. Here we address the problem of antigen heterogeneity and loss by designing bispecific hybrid receptors composed of two scFvs of different specificities, one fused to the TCRa constant chain and the second scFv fused to TCRb chain. Bispecific hybrid receptors for pairs of myeloma antigens (BCMA/SLAMF7; BCMA/GPRC5D; BCMA/CD229) were expressed in primary T cells and bind each of the cognate antigens. We used base editing technology to edit the endogenous TCR chains and avoid mispairing with our synthetic receptors, and to edit SLAMF-7, which can be expressed in T cells and cause fratricide by T cells expressing a SLAMF-7 targeting receptor. Bispecific TCR/CAR receptors expressed well in primary T cells and conferred T cells with target specificity as they produced cytokines, proliferated and demonstrated cytotoxicity after co-culture with target cells expressing BCMA and SLAMF-7 antigens. T cells equipped with our novel bispecific receptors maintained functions when exposed to MM1-R BCMA^{ko} or SLAMF-7^{ko} while T cells expressing a mono-specific receptor failed to kill targets demonstrating that both antigen binding domains can be selectively engaged and trigger T cell functions against targets expressing low antigen levels. Furthermore, presence of both target antigens on the tumor cells provided increased functional avidity measured by z-movi assay, increased Ca2+ flux and higher amounts of cytokines compared to tumor cell lines expressing a single antigen. Signaling studies demonstrated preserved superior antigen sensitivity in comparison to classical CAR design. *In*

vivo studies using NSG mice implanted with MM1-R^{WT} cells demonstrated similar anti-tumor efficacy for bispecific T cells compared to classical BCMA-specific CAR T cells but superior tumor control when mice were engrafted with a mixture composed of 85% MM1-R^{WT} and 15% MM1-R^{BCMAko} cells mimicking target antigen expression heterogeneity and antigen loss settings.

Bispecific hybrid T cell receptors embedded with natural TCR signaling machinery represent a promising therapeutic option to address antigen downmodulation/loss and antigen heterogeneity for treatment of MM.

Negative impact of the GABA pathway on αPD-1/PD-L1 immunotherapy

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While check-point inhibitor therapies have shown efficacy in the treatment of solid tumors, not all patients respond to treatment, suggesting that genetics, tumor microenvironment, and other host related or environmental factors may have bearing on this response.

To better understand the underlying mechanisms of resistance to anti-PD-L1 treatment, we performed unbiased transcriptional analysis of human tumors in responders and non-responders to anti-PD-L1 therapy. Intriguingly, tumors from non-responder patients were enriched in genes associated with the transport and the function of the neurotransmitter gamma-amino butyric acid (GABA) compared to responders. GABA is the main inhibitory neurotransmitter in the central nervous system but it's role in the periphery as an immunomodulatory agent is less well understood. We examined here the impact of GABA on response to immunotherapy in preclinical tumor models and analyzed the association with clinical outcomes in 2L cancer patients treated with immunotherapy.

We showed that important genes controlling the GABA pathway are enriched in human tumors versus normal tissue across indications, and tumor cells themselves are able to produce GABA. We found that several key genes of the GABA pathway are upregulated in non-responders to anti-PD-L1 and we derived a 5-gene signature associated with poor outcomes in 2L urothelial cancer patients receiving anti-PD-L1. Preclinical models further indicated that GABA at the

tumor site promotes tumor growth and dampens adaptive immunity preventing response to immunotherapy.

Given the widespread use of positive modulators of GABA-A receptors as palliative care in clinical practice, we then evaluated the association of GABA-A receptor agonists usage with outcomes in patients receiving checkpoint blockade. Strikingly ~45% of urothelial cancer patients received concomitant GABA-A positive allosteric modulators in anti-PD-L1 trials IMvigor-210 and -211. This group exhibited significantly lower OS and PFS as compared to anti-PD-L1 treated patients who did not receive GABA-A positive modulators. A similar negative impact was seen in patients receiving standard of care anti-PD1 and anti-PD-L1 immunotherapy in 2L NSCLC using real-world data from deidentified Flatiron Health - Komodo Health claims.

Further evaluation of concomitant GABA-A receptor agonists use will be required to better understand mechanism of resistance to checkpoint blockade and potential risks associated with palliative care in the setting of cancer immunotherapy.

Neoantigen targeted therapy promotes expansion of CD4+ T follicular helper cells and induces rejection of established tumors in mice

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Neoantigen-specific $CD4^+$ T cells have been shown to contribute to effective anti-tumor immune responses. Indeed, vaccine-induced or adoptively transferred neoantigen-specific $CD4^+$ T cells are capable of controlling tumor growth in both preclinical models and cancer patients. However, the mechanisms underlying tumor control by tumor-specific $CD4^+$ T cells remain unclear.

In this study, we used the E0771 preclinical model, for which we demonstrated complete rejection of established tumors post vaccination with an RNA-Lipoplex (LPX) molecule encoding an MHC-class II (MHCII)-restricted neoantigen expressed by tumor cells. Using a peptide:MHCII

tetramer to track neoantigen-specific $CD4^+$ T cells in mice bearing E0771 tumors, we show that $CD4^+$ T cells with Tfh, Th1, and Treg phenotypes recognized the same neoantigen in the spleen and tumor site in untreated tumor-bearing mice. RNA-LPX immunization induced a greater accumulation of neoantigen-specific $CD4^+$ Tfh cells in the tumor and tumor rejection only when the RNA-LPX encoded the relevant neoantigen. We further demonstrated that $CD8^+$ T cells, B cells, and macrophages were required for anti-tumor efficacy.

These findings suggest that anti-tumor activity of MHCII-restricted neoantigen-specific therapy relies on neoantigen expression by tumor cells as well as specific $CD4^+$ T cell phenotypes involved in orchestrating a multifaceted anti-tumor immune response.

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NK-cell educating KIR/HLA combinations impact survival in anti-PD-L1 treated cancer patients

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Natural killer (NK) cells are educated through the binding of killer immunoglobulin like receptors (KIR) with human leukocyte antigen (HLA) proteins, but it is currently unknown whether the presence of these highly diverse KIR-HLA interactions influences responses to immunotherapy in solid tumors. We make two observations that shed light on NK cell function and abundance in anti-tumor immune responses. In a meta-analysis of three clinical trials including 954 patients with non-small cell lung cancer (NSCLC) treated with the anti-PD-L1 antibody atezolizumab (IMpower150, IMpower130, IMpower131), we found that individuals who carried HLA-C1 alleles and the copy-number variable gene coding for its receptor *KIR2DL3* showed improved overall survival (OS) [$P = 5.0x10^{-5}$, HR=0.69]. In addition, the combination of *KIR3DL1* and HLA-Bw4 was nominally significantly associated with improved OS [P= 0.02, HR=0.82]. We hypothesized that the presence of both *KIR2DL3*/HLA-C1 and *KIR3DL1*/HLA-Bw4 might result in improved outcome

when compared to groups of patients with only one or none of the two combinations. We observed the strongest separation in a comparison of groups of patients with both vs. none [P = 0.002, HR = 0.59; median OS difference of 13.2 months in the IMpower150 study].

Consistent with other recent publications, we could not replicate earlier associations of specific HLA types, heterozygosity, or evolutionary divergence with outcome.

In addition to NK cell reactivity, their infiltration into tumor tissue could be an important determinant of patient outcomes. In 371 patients in IMpower150 treated with atezolizumab, an above median NK cell score was associated with longer OS [P = 0.008, HR = 0.73], and patients who had a high NK cell score and carried *KIR2DL3/*HLA-C1 showed the best overall survival, significantly different from patients with low NK cell infiltration and without the KIR/HLA combination.

These findings, based on a human genetics framework and thus establishing a causal relationship, might have implications for clinical trial design, and in combination with other tumor intrinsic and extrinsic factors contribute to statistical models better predicting patient outcomes.

Non-canonical regulatory Roles of Natural killers during tumor immune escape

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Objective responses to immune therapies such as CAR-T cells and immune checkpoint blockades are largely influenced by the tumor microenvironment (TME). While Natural Killer (NK) cells are best known for their cytotoxic functions, several recent studies have established the key involvements of NK cells in the modulation of the TME. Our previous study was amongst the first to characterize how tumor cells could hijack NK cells to express CD73 and acquire a regulatory phenotype (Neo et al. JCI. 2020). Later on, it was also discovered that differential activation of NK cells resulted in the modulatory effects on myelopoiesis of the tumor-bearing host (Neo et al. JITC. 2022). While NK cells could cross-activate dendritic cells to prime the TME for favorable responses to conventional immune therapies, we demonstrated that NK cell-derived IL-6 potentiated the development of myeloid suppressor cells (MDSCs) to induce

immune tolerance. With both NK-secretome profiling and single cell transcriptomics, we continue to uncover novel regulatory roles of intratumoral NK cells from both liver and ovarian cancers to identify several key chemokines that would be critical for homing of both myeloid and T cells during anti-tumor responses. With our current interest to consider how NK cells would influence host's responses to adoptively infused CAR-T cells, a panel of immune-modulating compounds were also screened for their effects on NK cells as potential therapeutic candidates to complement CAR-T cell therapies. Taken together, our findings highlighted the significance of regulatory NK cells and provided critical insights to circumvent immune resistance particularly in solid tumors.

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Nonreciprocal plasticity and divergent development of tissue resident memory and exhausted CD8+ T cells

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Cancer and chronic infections promote the development of exhausted $CD8^+$ T (T_{EX}) cells that fail to combat disease progression. While T_{EX} cells are distinct from conventional central and effector circulating memory T (T_{CIRCM}) cells, they share many overlapping features with non-circulating tissue-resident memory (T_{RM}) cells that develop after acute infection. $CD8^+$ T cells that co-express markers of terminal exhaustion and the hallmark tissue-residency markers CD69 and CD103 are abundant in many solid cancers where they correlate with favorable immunotherapy responses and are often classified as T_{RM} cells. However, similarities between T_{EX} and T_{RM} cells make it difficult to interpret $CD8^+$ T cell identity and lineage dynamics in the context of persisting antigen. Here, we used the lymphocytic choriomeningitis virus (LCMV) model of acute-resolving and chronic infection to interrogate the developmental relationships between committed T_{CIRCM}, T_{RM} and T_{EX} cells. We found that T_{CIRCM} cells, non-epithelial $CD103^-$ and epithelial $CD103^+$ T_{RM} cells could all become exhausted and give rise to heterogeneous

subsets of T_{EX} cells including $TCF1^+$ progenitor cells during chronic antigen stimulation. Conversely, established T_{EX} cells persisted in the circulation but inefficiently upregulated markers of tissue-residency including CD103 and CD49a in peripheral tissues after antigen withdrawal. Nevertheless, chronic antigen stimulation drove T_{EX} progenitors to seed a distinct subpopulation of terminally exhausted $CD39^{hi}CD69^+CD103^+$ $CD8^+$ T cells resembling *bona fide* T_{RM} cells in most peripheral tissues. Simultaneous cell-surface, transcriptional and epigenetic profiling of $CD8^+$ T cells from matched tissues during acute and chronic infection revealed these peripheral T_{EX} cells were fundamentally distinct from their T_{RM} counterparts and from T_{EX} subpopulations residing in secondary lymphoid organs. Our results highlight unappreciated heterogeneity in the terminal T_{EX} cell pool and imply $CD69^+CD103^+$ T_{EX} and T_{RM} cells are disparate cellular lineages with unilateral plasticity. These findings have implications for interpreting the immune landscape of solid tumors and determining the relative contributions of $CD8^+$ T_{EX}, T_{RM} and T_{CIRCM} cells to cancer immunotherapy responses.

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Notch signaling prevents severe exhaustion of CD8 T cell

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During chronic infection, the persistence of antigen and inflammation leads to CD8 T cell exhaustion, a state characterized by expression of inhibitory receptors (IRs), such as PD-1, and loss of functionality. Different subsets of exhausted CD8 T (Tex) cells are generated following chronic infection. Tex progenitors expressing the transcription factor TCF-1 give rise to Tex effector-like cells expressing CX3CR1, which have the best effector functions among Tex subsets. Tex effector-like cells become terminally differentiated Tex cells expressing CD101. PD1/PD-L1 blockade reinvigorates the progenitor subset to generate Tex effector-like cells, but the molecular events controlling Tex cell subset differentiation are still poorly defined. As Notch

signaling may be sustained during chronic infection by persistent TCR stimulation and inflammation, we postulated that it will influence Tex cell differentiation. To test this, we studied the immune response to LCMV clone 13 infection in mice with or without *Notch1/2* expression in mature CD8 T cells. Following infection, *Notch1/2*-deficient Tex cells expressed higher levels of IRs and were less functional than *Notch1/2*-sufficient CD8 T cells. In the absence of Notch signaling, Tex progenitors and terminally differentiated Tex cells accumulated, while Tex effector-like cells were depleted. Despite accumulation of Tex progenitors, *Notch1/2*-deficient Tex cells were not reinvigorated functionally by anti-PD-L1 treatment. Adoptive cell transfer experiments showed that Notch controls the differentiation of Tex progenitor into Tex effector-like cells. Analysis of the transcriptome of *Notch1/2*-deficient Tex cells suggests that Notch signalling acts by regulating Tex cell responsiveness to cytokines and CD4 help. Finally, we showed that Delta-like1 and 4 Notch ligands expressed by *Ccl19-Cre*$^{+}$ lymphoid tissue fibroblastic reticular cells were necessary during all stages of the infection to prevent severe exhaustion. Manipulation of Notch signalling may help to improve CD8 T cell response during chronic infection and cancer. *Funded by CIHR*

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PD-1 regulates tumor-infiltrating CD8 T cells in both a cell-intrinsic and cell-extrinsic fashion

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PD-1 pathway blockade has led to improved clinical outcomes in diverse cancer types, and has been approved by the FDA for use in over 20 different cancers. Although PD-1 pathway inhibitors have shown great promise, the mechanisms contributing to protective anti-tumor immunity following loss of PD-1 signals remain incompletely understood. To elucidate the cell-intrinsic consequences of PD-1 loss as well as the impact of PD-1 deletion on PD-1-expressing $CD8^+$ T cells in the same tumor microenvironment (referred to as cell extrinsic effects), we developed an inducible PD-1 knockout (KO) model whereby PD-1 could be deleted on about half of the cells. Inducible deletion of PD-1 beginning at day 7 post-implantation of MC38 tumor cells subcutaneously was sufficient to induce robust anti-tumor immunity and tumor control. Remarkably, PD-1-expressing $CD8^+$ T cells in the tumor had increased functionality similar to the T cells lacking PD-1. Using paired single cell RNA seq and TCR seq, we found that many of the transcriptional changes following PD-1 deletion were not restricted to $CD8^+$ TILs that lost PD-1, but instead shared by both PD-1-deleted and PD-1-expressing $CD8^+$ T cells. While a cell-intrinsic loss of PD-1 resulted in increased clonal expansion on a clone-by-clone basis, major transcriptional (e.g., increased cytotoxicity, IFN stimulation, and exhaustion) and functional changes were not restricted to $CD8^+$ TILs that had lost PD-1. These data provide additional mechanistic insight into how loss of PD-1 signals promotes protective anti-tumor immunity, indicating that PD-1 inhibitors can act beyond each individual cell that they contact to promote enhanced T cell function. These findings have important implications for design of PD-1-based therapeutic strategies in cancer patients.

PD-L1 checkpoint blockade promotes regulatory T-cell activity which underlies therapy resistance

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Immune checkpoint blockade (ICB), especially inhibition of PD-1 or its ligand PD-L1, has provided a paradigm shift in cancer treatment by unleashing anti-tumor immunity. Although ICB

has shown superiority compared to more conventional therapies for multiple types of cancer, the majority of treated patients do not or only temporarily respond for reasons incompletely understood. Regulatory T cells (Tregs) are an important suppressive immunocyte and are able to express high levels of PD-1 and could therefore be involved in dictating response to treatment with PD-1/PD-L1 blockade. However, to date, the effect of ICB on Treg function and the relation to treatment resistance has not been fully clarified. Identification and subsequent targeting of this role could overcome resistance to ICB to eventually improve immunotherapy efficacy.

To assess the effect of anti-PD-L1 on T-cell subsets (CD8 T cells, CD4 T helper cells and Tregs), we performed spatiotemporal analyses in the AE17-OVA mesothelioma murine tumor model, which were validated in the MC38- and B16F10 model using multicolor flowcytometry. We further validated the effect on Tregs by performing RNA sequencing and in vitro suppression assays. Furthermore, Tregs were depleted using anti-CD25 to investigate the functional involvement in treatment resistance. For clinical importance, single-cell RNA sequencing (scRNA seq) data obtained from Tregs isolated from human skin cancer patients (n=11) prior to and after anti-PD-1 treatment were reanalyzed (Yost et al., Nat Med, 2019). In addition, peripheral blood obtained from lung cancer (n=28) and mesothelioma patients (n=15) was assessed for the activation of Tregs following ICB.

We found that Tregs were primarily activated in treatment resistant murine tumor models compared to CD8 T cells and CD4-Th cells. This was reversed in treatment responsive models, where we found a profound activation of CD8 T cells and only a marginal effect on Tregs. When further dissecting the effect of anti-PD-L1 treatment on Tregs, we revealed that Tregs increase their immunosuppressive capacity on protein, gene and functional level following treatment in the treatment resistant AE17-OVA model. Treg depletion by using a Fc-optimized anti-CD25 antibody sensitized anti-PD-L1 resistant AE17-OVA and B16F10 tumors by delaying tumor growth and prolonged survival compared to both monotherapies. This coincided with strong upregulation of proliferation (assessed by Ki67) and activation (IFN□/TNFα/PD-1) by CD8 T cells and CD4-Th cells in peripheral blood. Finally, we also showed the clinical importance of Tregs in resistance to ICB as scRNA seq data revealed upregulation of a suppressive transcriptional gene program post ICB treatment, which was associated with lack of treatment response. In addition, anti-PD-1/PD-L1 ICB induced PD-1^{+} Treg activation in peripheral blood of lung cancer and mesothelioma patients which was especially pronounced in non-responders.

In conclusion, these data reveal a yet unappreciated role for Tregs underlying anti-PD-1/PD-L1 treatment resistance, thereby providing guidance for identification of novel therapeutic targets aimed at rewiring Tregs to improve anti-PD-1/PD-L1 efficacy.

PD-L1 regulates inflammatory macrophage development from human pluripotent stem cells via interferon-gamma signal

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PD-L1 (programmed death-ligand 1) serves as a pivotal immune checkpoint in both the innate and adaptive immune systems. Not only in cancer cells but PD-L1 is also expressed in macrophages in response to interferon-gamma (IFNγ). We hypothesized that PD-L1 might regulate macrophage development. We established *PD-L1*$^{-/-}$ human pluripotent stem cells by CRISPR-targeting. We differentiated them into macrophage lineage with Stemdiff media, M-CSF, LPS, and IFNγ. We observed a 60% reduction of CD11B^{+}CD45^{+} macrophages in *PD-L1*$^{-/-}$, orthogonally verified with PD-L1 inhibitor BMS-1166 reduced macrophages to the same fold. Single-cell RNA sequencing further confirmed the 60% reduction of macrophages as well as the down-regulation of macrophage-defining transcription factors SPI1, MAFB, and KLF6. Further, *PD-L1*$^{-/-}$ macrophages reduced the level of inflammatory signals such as NFκB, TNF, and chemokines CXCL and CCL families. Whilst anti-inflammatory TGF-β was exclusively upregulated. Cell Phone analysis revealed an increase in collagen score, an indicator of anti-inflammatory macrophages. Finally, we identified that *PD-L1*$^{-/-}$ macrophages significantly down-regulated interferon-stimulated genes (ISGs) despite the presence of IFNγ in the media. Indeed, *PD-L1*$^{-/-}$ macrophages lost IFNGR1^{+} populations (70% vs 91% in *wild type*), explaining that cells could not respond to IFNγ. These data suggest that PD-L1 is a necessary regulator of IFNγ signal and subsequent inflammatory macrophage development. Consistently, the phase II trial of PD-L1 inhibitor durvalumab in patients with advanced esophageal and gastroesophageal junction adenocarcinoma increased the proportion of anti-inflammatory macrophages to pro-inflammatory ones in the tumor (NCT02639065, Mamdani *et al*., *Front in Onc*. 2021). Our results suggest the potential clinical significance of the increase of anti-inflammatory macrophages in PD-L1 inhibitor treatment.

Personalized identification of neoantigen-specific TCRs using synthetic TCR library screens

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T cell receptor (TCR) gene therapy is a promising form of cellular immunotherapy where peripheral blood T cells of cancer patients are genetically engineered with tumor-specific TCRs *in vitro* before being reinfused into the patient. Contrary to immune checkpoint blockade and tumor-infiltrating lymphocytes (TIL) therapy, TCR gene therapy does not rely on a pre-existing intratumoral T cell response, and therefore holds promise for the treatment of less immunogenic cancers that respond poorly to current immunotherapies. Accumulating evidence suggests that T cell activity towards neoantigens – antigens that arise as a result of tumor mutations – is an important driver of anti-tumor T cell immunity, and there are strong efforts that aim to exploit neoantigen-specific TCRs for TCR gene therapy. However, the identification of such TCRs is complicated by both the patient-specific nature of tumor mutations, and the fact that these TCRs may be exceedingly rare.

Here, we developed a personalized TCR discovery pipeline that combines large-scale assembly of synthetic TCR libraries with functional genetic screening, enabling the profiling of 1,000s of individual TCRs in a single experiment. In this approach, T cells engineered with patient-derived TCR libraries are cocultured with matched antigen-presenting cells (APC) that are modified to express the patient's tumor mutanome, and responding TCRs are identified by next-generation sequencing. Since the patient APCs are fully MHC class I- and class II-proficient, this enables the unbiased functional screening of both MHC-I- and MHC-II-restricted patient T cell repertoires.

We leveraged the method to screen ~3700 TIL-derived TCRs from three patients and identified dozens of neoantigen-specific TCRs. Notably, these included multiple neoantigen-specific TCRs identified in an ovarian cancer patient with a low mutational burden. In TCR transfer experiments, identified TCRs mediated cytotoxicity towards neoantigen-positive APCs and patient tumor cells. Correlating the TCR specificity back to the phenotype of their parental T cells *in situ* indicated that a number of neoantigen-specific clonotypes scored relatively low on recently identified tumor reactivity-predicting gene signatures, underscoring the importance of identification of therapeutic TCRs using truly functional screening assays.

Collectively, these data demonstrate the feasibility of personalized and high throughput discovery of neoantigen-specific TCRs, and the presented technology should catalyze the development of neoantigen-targeted TCR therapy.

Phenotypic differences in the tumour-associated macrophages between viral and non-alcoholic fatty liver disease (NAFLD) related hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) typically develops on a background of chronic inflammation, with risk factors including HBV/HCV and increasingly non-alcoholic fatty liver disease (NAFLD). Indeed, different HCC aetiologies may influence the tumour microenvironment (TME) with implications for immunotherapy response, such as immune checkpoint inhibitor therapy. Emerging evidence suggests a poorer immunotherapy response for NAFLD-HCC versus viral HCC, yet no therapies exist for NASH and associated liver fibrosis. Macrophages are the major PD-L1 expressing cells in the liver microenvironment, and their pro-inflammatory or anti-inflammatory functions directly influence the disease progression from hepatic inflammation to malignancy. We have characterised the tumour associated macrophages (TAMs) in samples of viral vs NAFLD-related HCC. For viral-HCC, while M2 macrophages are low in both non-tumoural liver (NT) and tumour (T), the tumour has significantly lower proportion of M1 macrophages compared to NT. The situation is reversed for NAFLD-HCC, where both NT and T possess few M1 macrophages and high M2 macrophages, though the tumour has higher M2. Interestingly, the PD-L1+ macrophages are high in the viral NT and low in T. This is yet again reversed in NAFLD-HCC samples, where the PD-L1+ macrophages are low in NT and high in T. With an *in vitro* liver organoid model that comprises iPSC-derived hepatocytes, Kupffer cells, hepatic stellate cells, and HCC cancer cells, we have recapitulated the TAM phenotypes observed in viral and NAFLD-related HCC patient samples. Using an organoid-T cell co-culture model, we have shown that the above phenotypic differences directly contribute to T cell dysfunction. Taken together, these results show that the phenotypes of liver macrophages are dependent on the disease aetiology. Compared with viral hepatitis, NAFLD leads to more M2 yet fewer PD-L1+ macrophages. Though it is the opposite in their respective tumours, a high-M2, low-PD-L1+ macrophage population

may impede T cell function in checkpoint inhibitor therapy. Further studies will address the macrophage-T interaction in more detail, leveraging both patient samples and *ex vivo* and *in vitro* organoid models.

PHGDH-mediated Endothelial Metabolism Drives Glioblastoma Resistance to CAR T cell Immunotherapy

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Immunotherapy by adoptive transfer of engineered T cells holds promise for treating solid tumors. Still, its efficacy is limited by the paucity of T cells infiltrated into the tumors through aberrant tumor vasculature. The mechanisms underlying vascular aberrancy, particularly regulated by endothelial cell (EC) metabolism, remain largely unknown. Here, we report that PHGDH-mediated EC metabolism fuels the formation of a hypoxic and immune-hostile vascular microenvironment, inducing glioblastoma (GBM) resistance to chimeric antigen receptor (CAR)-T cell immunotherapy. Our single-cell transcriptomic and metabolomic analyses of human and mouse GBM tumors identify that PHGDH, a key serine synthesis enzyme, is preferentially upregulated in tumor ECs, whose expression is driven by microenvironmental stress via ATF4. We reveal that PHGDH induces serine-dependent nucleotide and glutathione biosynthesis, leading to redox-dependent glycolysis that facilities EC proliferation and vascular over-sprouting. Notably, genetic ablation of PHGDH prunes tumor vasculature, relieves hypoxia, improves T cell infiltration, and extends survival in GBM-bearing mice. PHGDH inhibition activates anti-tumor T cell immunity and sensitizes tumors to CAR-T cell therapy. Together, our findings uncover that PHGDH-mediated serine metabolism is a mainly altered metabolic pathway in tumor ECs, which induces aberrant vascularity. Reprogramming endothelial metabolism by targeting PHGDH may, therefore, serve as a promising strategy for reconditioning the immune-vascular microenvironment to overcome solid tumor resistance to T cell-based immunotherapy.

Predicting immune cell abundance and gene expression in the tumor microenvironment from blood single-cell transcriptomics

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The tumor microenvironment (TME) is a key determinant of immunotherapy efficacy, yet, not readily accessible. Here, we ask if the immune cell fractions (ICFs) and gene expression patterns in the TME can be predicted from the blood single-cell RNA sequencing (scRNA-Seq) data. To the best of our knowledge, the immune status of the TME has not been comprehensively evaluated and predicted from the blood previously. Addressing this challenge, we analyzed matched blood and tumor samples from 26 head and neck squamous cell carcinoma patients with CD45+ scRNA-Seq data and clinical information. We developed a machine learning predictor of ICFs and gene expression levels of major immune cell types in the TME from the matching blood transcriptomics and pertaining clinical information. We find that the ICFs of all 11 major immune cell types in the TME were predictable from the blood; ~ 20 - 50% genes expressed in different immune cells were predictable from the blood, with the highest coverage in dendritic cells and helper, cytotoxic and regulatory T cells. Furthermore, the ICF ratio signature of $(B_{memory} - T_{reg}) / (B_{memory} + T_{reg})$ in the TME, which can be precisely learned from the blood, can robustly predict not only the response to immune checkpoint blockade (ICB; AUC = 0.72-0.79, p = 0.004-0.05), but also the survival of patients after ICB treatment (overall survival, p = 0.05-0.1; progression-free survival, p = 0.01-0.03), on both single-cell and bulk expression datasets. These results are the first to demonstrate the feasibility of employing single-cell transcriptomics liquid biopsy to predict the immune status in the TME of cancer patients, facilitating future personalized cancer precision therapy.

Progesterone Receptor Modulates the Antigen Processing and Presentation Machinery Decreasing MHC Class I Expression on Mammary Gland Tumors

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Hormone receptor (HR)-positive breast cancers, which account for approximately 75% of all breast cancers and express both estrogen receptor (ER) and progesterone receptor (PR), have been the subject of much research. As such, successful anti-estrogen/ER-based therapies have been very successful in the treatment of HR+ breast cancer. Despite this success, over 1/3 of patients will eventually progress on current ER/estrogen-targeted therapies, underscoring the need for new therapies in HR+ breast cancer. Unlike ER, the role of PR in breast cancer progression has received far less attention. In a previous study, we found that the mouse mammary tumor cell line E0771, modified to express the mouse progesterone receptor (mPR) and the OVA peptide, demonstrated decreased T cell-mediated cell death when treated with progesterone compared to the control group. Progesterone-mediated protection from T-cell death was only observed in mPR+ cell lines. Flow analysis of the E0771-OVA-mPR cells also revealed a decrease in MHC Class I presentation in progesterone-treated cells compared to vehicle control. To further explore the role of the machinery required for antigen processing and presentation of MHC Class I molecules, we conducted additional experiments using the E0771 cell line. Our results showed that treatment with progesterone led to a reduction in RNA levels of all the machinery required for antigen processing and presentation (APP), including Tap1, Tap2, Tapbp, Nlrc5, B2m, and Psmb8, which are essential for APP. Based on these findings, we propose that activation of the progesterone receptor by progesterone may decrease MHC Class I expression by regulating APP. The findings of this study may have significant implications for our understanding of how breast cancer cells evade T-cell mediated cytotoxicity.

Pyroptosis activated conventional type I dendritic cells mediate the priming of highly functional anticancer T cells

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Initiation of anti-tumor immunity is reliant on the stimulation of dendritic cells (DCs) to present tumor antigens to naïve T cells and generate effector T cells that can kill tumors. Induction of immunogenic cell death during chemoradiotherapy can stimulate T cell-mediated immunity. However, many cytotoxic anticancer therapies simultaneously activate multiple types of cellular stress and programmed cell death; hence, it remains unknown what types of cancer cell death confer superior antitumor immunity. We explored whether induction of pyroptosis, a form of immunogenic cell death, is superior to apoptosis in activating DCs for anti-tumor immunity. Using an inducible system whereby the effect of different forms of cell death could be studied in isolation, we found that, unlike apoptosis, pyroptosis induces an immunogenic pro-inflammatory transcriptome and secretome signature. In both prophylactic and therapeutic contexts pyroptosis preferentially activated CD103+ type I conventional DCs (cDC1) to prime a higher magnitude of anti-tumor immunity through expansion of tumor-specific CD8+ T cells. Depletion of cDC1 or CD4+ and CD8+ T cells ablated the anti-tumor response, leaving mice susceptible to tumor rechallenge. Thus, developing therapeutics that induce pyroptosis may serve to improve anti-tumor immune responses.

Rational adjuvant design using self-assembly of immune signals to program innate immunity and anti-tumor response

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Current cancer immunotherapies rely on specific stimulatory cues to generate immune response. Interestingly, many recent clinical studies show activating multiple innate immune pathways drives robust, biased responses during cancer. Biomaterials offer useful features to deliver multiple cargos, but add translational complexity owing to intrinsic immunogenic properties. We designed a modular adjuvant platform using self-assembly to build nanostructured capsules comprised entirely of either model or human melanoma antigens,

along with multiple classes of toll-like receptor agonists (TLRas). These assemblies sequester TLR to endolysosomes, allowing programmable control over the relative signaling levels transduced through these receptors. Excitingly, combinatorial control of innate signaling allowed generation of distinct adaptive immune responses against a given antigen by controlling the ratio of agonists self-assembled with antigen. These assemblies drive reorganization of lymph node stroma to a pro-immune microenvironment, expanding antigen-specific T cells. Excitingly, assemblies built from antigen and multiple TLRas enhance T cell function and anti-tumor efficacy compared to ad-mixed formulations or capsules with a single TLRa. Finally, capsules built from a clinically relevant human melanoma antigen and up to three TLRa classes enable simultaneous control of signal transduction across each pathway. This creates a facile adjuvant design immunotherapy platform to tailor signaling. The modular nature supports precision juxtaposition of antigen with agonists relevant for several innate receptor families, such as toll, STING, NOD, and RIG.

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Regulatory T cell depletion unleashes an anti-tumor IFNγ+ γδ T cell response in the tumour microenvironment

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γδ T cells are potent anti-tumor effectors, capable of orchestrating immune responses that control tumour progression. The anti-tumor properties of γδ T cells are mostly attributed to the fast and strong production of IFNγ. However, the cellular and molecular regulators of IFNγ-producing γδ T cells in the tumour microenvironment (TME) are largely unknown. Here, we used a murine orthotopic model of breast cancer (E0771) implanted in a FOXP3-DTR transgenic mouse strain, in which we were able to deplete regulatory T (Treg) cells upon diphtheria toxin administration, to study how Treg cells affect γδ T cell behavior in the TME. We showed that mice Treg cell depletion increased accumulation of specifically anti-tumoral IFNγ-producing γδ T cells and not IL-17-producing γδ T cell subsets in the TME, which was accompanied with decelerated tumor growth. Strikingly, this depletion also led to accumulation of IFNγ-producing ab T cells in the TME. We demonstrated that Treg cells specifically inhibit proliferation of IFNγ-producing γδ T cells in vitro and in vivo. We observed that the TME induced the expression of regulatory molecules on Treg cells as well as the release of IL-13 and IL-10. In parallel, IFNγ-committed γδ T cells express high levels of the IL-13 receptor and IL-10 receptor. Blockade of IL-10 and IL-13 impairs the inhibition of IFNγ-producing γδ T cells by Treg cells. This work identifies

a novel axis by which Treg cells specifically target anti-tumor γδ T cell subsets in the TME. Our findings open opportunities for the design of new therapeutic strategies to boost effective anti-tumour responses.

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Remodeling Tumor Microenvironment with Chimeric Antigen Receptor iMacrophages (CAR-iMac)

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Immunosuppressive tumor microenvironment (TME) impairs chimeric antigen receptor (CAR)-immunocytes in solid tumors such as hepatocellular carcinoma. Leveraging the robust generation of functional macrophages (iMac) from human pluripotent stem cells, we established CAR-iMac. iMac sustained over two weeks without changing the CD80 inflammatory gene level in culture, hinting at the feasibility of engineering and subsequent application. We target "do-not-eat-me" signals by replacing the loci of *SIRPA* and *SIGLEC10* with the CAR cassette. The resultant CAR-iMac is resistant to cancers' do-not-eat-me signals. We found that human hepatocellular carcinoma cell line Huh7 expressed CD47 and CD24, ligands of the signals, rendering escape from macrophages. That indicates Huh7 is a suitable model to measure CAR-iMac's resistance to do-not-eat-me signals. We engineered CAR-iMac by secreting IL12 and IL18, leading to remodeling TME into the tumoricidal niche. And we confirmed that IL12 and IL18 induced NK cells to secrete interferon-gamma for cytotoxicity. We anticipate that CAR-iMac will enhance tumor-killing by immune priming NK cells. Altogether, our strategies for robust generation of functional macrophages, targeting do-not-eat-me signals, and immune priming leverage the amenable feature of human pluripotent stem cells in cancer immunotherapy.

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Reprogramming myeloid cells by JAK inhibition to enhance checkpoint blockade immunotherapy

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Hypothesis: Checkpoint inhibitors have achieved a wide adoption for treatment of advanced cancer, but the majority of patients fail to respond. Among the underlying causes are redundant mechanisms driving cytotoxic lymphocyte dysfunction. Motivated by the discovery of JAK inhibitors as hit compounds in a screen for the reversal of T cell exhaustion, we hypothesized that JAK inhibition could enhance the efficacy of checkpoint inhibitors in cancer.

Methods: We performed preclinical and clinical studies of the JAK inhibitor ruxolitinib in combination with immune checkpoint inhibitors (ICI) for the treatment of cancer. In murine immunocompetent syngeneic cancer models, ruxolitinib with anti-PD1 + anti-CTLA4 was evaluated for efficacy and biomarkers compared to ICI alone. Tumor-infiltrating, blood and lymphoid organ immune cells were phenotyped using single-cell transcriptomics, functional assays and flow cytometry. Clinically, the combination therapy was tested in an investigator-initiated Phase I/II clinical trial of ruxolitinib with nivolumab in relapsed or refractory Hodgkin lymphoma (NCT03681561). Patients who previously failed to respond to ICI received ruxolitinib for 1 week then nivolumab every 4 weeks concurrent with twice-daily ruxolitinib. Peripheral blood was collected at baseline, 1 week after ruxolitinib and 4 weeks after 1st nivolumab dose, and subjected to transcriptomic, functional and flow cytometry analyses.

Results: The ruxolitinib + ICI combination achieved better control of tumor growth than ICI in 3/4 of the tumor models examined (A20, LLC1, MC38). Remarkably, we observed a broad shift of tumor monocytes and granulocytes from a suppressive into an immunostimulatory state characterized by the expression of MHC molecules and the ability to stimulate T cell proliferation. Hodgkin lymphoma patients in the ruxolitinib with nivolumab trial exhibited an interim disease control rate of 76% (13/17). Ruxolitinib treatment in these patients did not impair T cell cytokine production but significantly reduced the neutrophil-to-lymphocyte ratio and the expression of myeloid derived suppressor cell markers in monocytes compared to pre-treatment. Complete responders showed a significantly greater reduction in the neutrophil-to-lymphocyte ratio and other myeloid metrics after ruxolitinib compared to progressive disease patients.

Conclusions: The combination of ruxolitinib with ICI was effective in preclinical models of cancer and in a Phase I/II Hodgkin lymphoma clinical trial. Unexpectedly, JAK inhibitor-mediated reprogramming of myeloid cells and the associated enhanced T cell activity may be important for the observed efficacy.

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Reprogramming the immune cells to stimulate an anti-tumor response using STING and TLR7/8 agonists for bladder cancer

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Bladder cancer (BC) is the 10th most common malignancy, affecting 578,000 people worldwide in 2020. The 5-year recurrence rate of BC approaches nearly 78%, necessitating life-long surveillance and making it the costliest cancer to treat. Though immunotherapies have revolutionized cancer treatment in general, improved therapy is needed for BC. The conventional therapy with Bacillus Calmette-Guérin (BCG) treatment has a low patient compliance and high relapse rate, while the checkpoint inhibitor atezolizumab only has a 20% response rate. These realities underscore the need to explore different therapeutic avenues to discover a durable BC therapy. We are evaluating the utility of combining STING and TLR 7/8 agonists to synergistically reprogram the TME by recruiting various subsets of immune cell to elicit a robust immune response against BC tumors. Using MB49 murine bladder cancer cell line and RAW 264.7 macrophages, we have evaluated the capacity of small molecule agonists to activate the TLR 7/8 and STING pathways, as well as polarize macrophages from an M2-like (pro-tumor) to M1-like (anti-tumor) status. These M1-like macrophages were co-cultured with MB49 cells to evaluate their cytotoxicity potential. Murine bone marrow derived antigen presenting cells like macrophages and dendritic cells were also tested for their activation and differentiation potential after agonist exposure. Our findings suggest that the combination of STING and TLR 7/8 agonists has the potential to reprogram immune cell populations to become anti-tumorous.

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Reprogramming the immune tumor microenvironment by targeting LILRB3

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Immune checkpoint blockade therapies, especially blocking antibodies that target the PD-1/PD-L1 pathway, have been successful in treating a wide variety of cancers. However, there is still a large portion of cancer patients in which the existing therapies are not effective. Myeloid-derived suppressor cells (MDSCs) and other immunosuppressive cells that inhibit anti-tumor immunity and support multiple steps of tumor development are recognized as one of the major obstacles in cancer treatment. The development of novel therapeutic strategies that reprogram the immunosuppressive tumor microenvironment might lead to better patient outcomes.

The leukocyte Ig-like receptor subfamily B (LILRB) receptors are a group of type 1 transmembrane glycoproteins with extracellular Ig-like domains that bind specific ligands and transduce inhibitory immune signals through intracellular immunoreceptor tyrosine-based inhibitory motifs. Based on our study demonstrating that LILRB3 is expressed on and support the activity of human MDSCs in cancer patients, we hypothesize that cancer cells induce the expression of LILRB3 on MDSCs which transduces inhibitory immune signals to support tumor growth. We developed a blocking antibody against LILRB3 which blocks ligand binding, prevents p-TYR/SHP1/SHP2 recruitment, and prevents inhibition of T cell proliferation by MDSCs *in vitro*. To study the role of LILRB3 *in vivo*, we created a myeloid-specific LILRB3 transgenic mouse model. We challenged the LILRB3 transgenic mice with various cancer cell lines and treated the mice with anti-LILRB3 blocking antibodies. Our analyses suggest that blocking LILRB3 signaling reduces tumor growth by reducing MDSCs and increasing T cell numbers.

Resident memory T cells in the lymph node balance localized and systemic protection

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Systemic metastasis is responsible for a majority of cancer-related deaths and the tumor draining lymph node (LN) is often a first site of dissemination and a poor prognostic across many solid tumor types. The endogenous mechanisms that might protect against LN metastasis during early tumor development remain poorly understood. LN resident memory T cells (Trm), which do not circulate, provide highly localized protection to cancer and correlate with better clinical outcomes. Here we track LN Trm development using a model of cutaneous viral infection (vaccinia) to determine the kinetics and anatomical requirements for their differentiation. We show that after cutaneous vaccinia virus infection, CD69+CD103+ Trm form in the draining LN coincident with their establishment at the initial site of infection in the skin.

LN Trm were found in various compartments of the LN including the sub capsular sinus which is the most common site for cancer metastasis. LN Trm formed specifically in LNs draining infected skin expressing their cognate antigen and did not form in contralateral controls. These data supported a hypothesis that T cell egress out of infected skin through lymphatic vessels was necessary for LN Trm formation. Consistent with this hypothesis, LN Trm were absent from mice lacking dermal lymphatic vessels indicating that either fluid transport or cellular transport from the skin to the LN was necessary for the establishment of LN Trm. To decouple the role of LN priming from T cell egress we resected the infected skin days 5, 13, and 20 post infection. Ear resection 5 days post infection completely impaired Trm formation despite the fact that circulating anti-viral immune responses were unaffected. Importantly, we demonstrated that LN Trm are poised for cell killing as evidenced by constitutive expression of granzymes, and were able to protect against viral rechallenge. These findings indicate that LN Trm are poised to provide rapid, localized protection specific to the draining LN. These data paired with our recent finding that tumor-specific CD8+ T cells also egress from the tumor may suggest that the sentinel LN harbors populations capable of controlling regional cancer metastasis.

Responders vs non-responders: Temporal transcriptomics during immune checkpoint therapy reveals dynamic activation of IFNβ determines response

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Background: Immune checkpoint therapy (ICT) results in complete regression of tumors in some patients, but no response in others. As the anti-tumor immune response is a constantly adjusting complex dynamic system, time-dependent mechanisms may underpin an effective response. However, it has not been possible to identify dynamic events in human studies due to the difficulty to sample the same tumor at multiple time-points during treatment. By understanding the dynamic processes underlying a successful response to ICT in mice, we hypothesized we could develop time-dependent combination treatments to improve efficacy.

Methods: We developed dual-tumor mouse models where ICT either leads to a response or a failure to respond in both tumors, allowing us to take one tumor at any time during ICT therapy whilst determining the response from the remaining tumor. In order to identify the biological processes that occur in tumors over the course of ICT, we mapped the gene expression of 144 responding and non-responding tumors from two mouse models at four time-points, using bulk and single-cell RNAseq, and we validated results on the protein level using flow cytometry and reporter mice.

Results: We found that responding tumors displayed a dynamic on/fast-off pattern of type I interferon (IFN) signaling, whilst in non-responsive tumors type I IFN was slowly and persistently activated. By mimicking the on/off IFN signal using recombinant IFNs and neutralizing antibodies, we were able to markedly improve the response to ICT, but only when IFNβ or its receptor were blocked, not IFNα. We identified Ly6C+/CD11b+/CCR2+ inflammatory monocytes as the primary source of IFNβ and found that active type I IFN signaling in tumor-infiltrating inflammatory monocytes was associated with T cell expansion in patients treated with ICT.

Conclusions: Our results show that when therapeutically targeting a dynamic process, a drug target may need to be modulated in a time-dependent biphasic manner in order to achieve optimal effect. This is the first example that time-dependent modulation of a drug target is required to achieve a more favorable outcome to ICT. With anti-IFNβ antibodies currently in development, these results can be rapidly translated into the clinic.

Robust prediction of pan-cancer immune checkpoint blockade response from genomic and clinical features using machine learning

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Introduction

Although immune checkpoint blockade (ICB) has revolutionized cancer treatment, the response rate is still low. Machine learning models that use multi-modal data to predict response are gaining attention. However, overfitting remains a challenge, particularly with small datasets, as machine learning models trained on such data may exhibit weaker performance on independent test data compared to the data they were trained on.

Methods

Our analysis involved the examination of 3496 patient samples spanning 18 solid tumor types from multiple cohorts. We employed up to 16 genomic and clinical features to construct various machine learning models, which we subsequently evaluated through standard cross-validation procedures and independent external datasets. We further assessed the models' ability to predict patient survival following immunotherapy, and moreover, their ability in predicting patient ICB response probability.

Key Results

- The Linear LASSO Regression (LLR) model exhibited the best performance, outperforming all other models with the highest AUC values on test sets. Particularly noteworthy is its minimal AUC difference between training and test sets, indicating excellent generalizability.
- The most significant predictive features are TMB, PD-L1 expression, whether chemotherapy was received prior to ICB treatment, blood albumin level, blood neutrophil-to-lymphocyte ratio, cancer type, and patient age.
- The LLR model consistently predicts overall survival and progression-free survival across all individual cancer types.
- Most importantly, ICB response probability increases near-monotonically from 0% to 100% with the LLR score, which will allow more precise patient stratification in future. In contrast, low-TMB patients (TMB < 5) still have an ICB response probability of

approximately 20%, and higher TMB does not always correlate with a higher response probability.

Conclusions

The new computational model based on LLR identifies crucial features that can predict pan-cancer ICB response and survival. Furthermore, utilizing a combination of genomic and clinical features has the potential to significantly enhance clinical ICB patient stratification beyond the conventional use of TMB.

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Sensitizing breast cancers to immune checkpoint inhibitors through CD27 agonism and vaccination against tumor-associated antigen

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Background: Responses to immune checkpoint inhibitors (ICIs) are variable, often due to a lack of pre-existing anti-tumor adaptive immunity. A potential means to elicit this type of immunity is through tumor antigen vaccination, which our group tested ~20 years ago using HER2 peptide and recombinant protein pulsed dendritic cells (DC vaccine) in six patients with refractory $HER2^+$ advanced or metastatic (stage II (≥ 6 +LN), III, or stage IV) Breast Cancer (BC). In 2019, we followed up with these patients and found that all six patients were still alive, 18+ years after vaccination. An in-depth analysis of blood samples from these patients using cytometry by time-of-flight (CyTOF) revealed a significantly increased presence of HER2-specific CD27+ expressing memory T cells, at 18+ years post-vaccination. These striking long-term memory T cell responses were congruent with recent studies demonstrating the importance of CD27 expressing memory T cells in long-term cellular immunity against SARS-CoV2 infection. Therefore, we hypothesized that CD27 plays a critical role in antigen-specific cellular immunity against BC, and the stimulation of CD27 expressing T cells with a CD27 agonist mAb could significantly increase the responses triggered by vaccination against tumor-associated antigens.

Results: In our studies, we first recapitulated the induction of memory $CD27^+$ antigen specific T cells among the vaccinated patients using a transgenic mouse model expressing human CD27. Using this model, we found that a single dose of human anti-CD27 antibody (Varlilumab) could significantly augment the induction of HER2-specific T cell responses after vaccination with a

HER2 recombinant vector, especially $CD27^+CD44^+$ memory CD4 T cells. Notably, we found that these Varlilumab-augmented responses persisted up to 120 days post vaccination. Contrary to some studies, both in vitro stimulation studies and in vivo CD4 depletion (during vaccine priming) demonstrated that direct CD27 stimulation of CD4+ T cells is critical for this effect. Notably, using an ICI-insensitive syngeneic $HER2^+$ BC models, we found 50% of mice in the combination group of anti-CD27 antibody plus HER2 vaccine showed total tumor regression by the end of study. When combined with anti-PD1 antibody, we found that the combination of HER2 vaccine and Varlilumab leads to total tumor regression in 90% of tumor bearing mice with syngeneic $HER2^+$ BC, indicating that the vaccination against tumor associated antigen HER2 plus anti-CD27 antibody sensitized ICI-insensitive $HER2^+$ BC toward ICI.

Conclusions: Our data demonstrates that the administration of anti-CD27 antibody significantly increases the long term presence of $CD27^+$ antigen specific memory T cells after vaccination against HER2. As a consequence, the combination of anti-CD27 with HER2 vaccination sensitized this immune unresponsive breast cancer to treatment with an anti-PD1 antibody. Our study suggests that the vaccination against tumor-associated antigen with mAb targeting CD27 leads to the robust cellular immunity, which is required for successful ICIs against HER2+ breast cancer.

Single cell atlas of microsatellite stable colorectal cancer studies and its use in drug discovery

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Colorectal cancer (CRC) is one of the most prevalent cancer worldwide. Little progress has been achieved to improve disease outcome for most of these patients in the past twenty years. Recent studies evaluating the impact of immunotherapy in colorectal cancer patients identified that patients with microsatellite instable tumors (MSI) have an extremely high rate of immunotherapy response (PD-1/PD-L1 blockade). However, this is a subgroup that accounts for 5-15% of patients. In addition to microsatellite instable patients, it is believed that a small percentage of microsatellite stable (MSS) patients may also benefit from immunotherapy, which has been recently published. To better understand the cellular and molecular features of microsatellite stable patients, we have collected eight publicly available single cell RNA sequencing datasets. We integrated all the studies using harmony and performed unbiased cell type annotation using a curated tumor cell type reference. Our CRC atlas consists of 166 tumor

patients (118 MSS, 48 MSI) and 97 matched normal samples. In addition to microsatellite status, we have anatomical information (left or right side tumors), mutational information and CMS status available while treatment status is known for a subset of samples. We have used this detailed cellular landscape to better understand the cellular landscape of clinically relevant patient groups.

Single cell multi-omic profiling of childhood Acute Lymphoblastic Leukemia (ALL) identifies a CD4 T-cell subset displaying features of dysfunction in DUX4-rearranged ALL

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Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy. It is caused by genetic and epigenetic alterations, resulting in uncontrolled expansion of abnormal B-lymphoid progenitor cells. ALL is classified into risk-stratifying subtypes based on specific somatic mutations. We and others have recently described a set of previously unrecognized subtypes, of which *DUX4*-rearranged (*DUX4*-r) ALL is among the most common. Current treatment schemes result in favorable outcomes for childhood ALL, but the cytostatic treatment is associated with significant toxicity and risk of long-term side effects. While immunotherapy based on chimeric antigen receptor T-cells targeting CD19 have shown high therapeutic efficacy, other immunotherapies are less effective, suggesting T-cell exhaustion or other immune escape mechanisms. Novel therapeutic options can potentially be identified by dissecting the cellular characteristics of ALL, and by defining the interaction of the malignant cells with the bone marrow (BM) microenvironment.

We used a multimodal single cell sequencing strategy to delineate the transcriptional and epigenetic characteristics of the cellular ecosystem of childhood ALLs (n=23) with a focus on the novel *DUX4*-r ALL subtype (n=11). The leukemic BM was observed to be severely altered with a massively expanded B-cell progenitor compartment displaying a distinct gene expression profile. The second most abundant cell type was T-cells. In general, they were indistinguishable from T-cells in normal BM. However, in *DUX4*-r ALL samples we identified a set of CD4+ T-cells, distinct from those found in normal BM. By performing differential gene expression analysis, GSEA and by studying cellular interactions using CellphoneDB, we identified transcriptional

features of the CD4+ T-cells, including both downregulation and upregulation of activation markers such as and *CCR7* (downregulated) and *TNFRSF9* (upregulated). Several anti- and pro-apoptotic markers, such as *PMAIP1* and *ROHB*, were highly upregulated. In addition, the CD4+ T-cells showed upregulation of genes involved in epigenetic enforcement of exhaustion, such as *BATF, FOS, JUN* and *NR4A*- and *TOX*-family genes. These transcription factors were further confirmed to display high activity specifically in T-cells from *DUX4*-r cases by motif footprinting of single cell ATAC-seq data. *LAYN* was the only T-cell inhibitory marker that was significantly upregulated compared to normal T-cells; however, markers such as *CTLA4*, *LAG3* and *PDCD1* were also expressed. Single-sample GSEA showed dysregulation of essential immune lymphocyte function gene sets such as “adaptive immune response”.

Since CD4+ T-cells are involved in B-cell maturation, we speculated that the aberrant CD4+ T-cells could interact with the leukemic B-cells. Interestingly, the CD4+ T-cells expressed *BCL6*, the lineage defining transcription factor for T follicular helper cell differentiation, and *CXCL13*, a chemoattractant for *CXCR5*-positive B-cells. In addition, *CXCL13* harbored open chromatin specifically in T-cells from *DUX4*-r ALL samples.

In conclusion, multimodal single cell profiling of *DUX4*-r ALL identified a distinct subset of CD4+ T-cells, displaying transcriptional and epigenetic features of dysfunction. Validation of these T-cells is currently ongoing, which might shed additional insights into the features of the population. These studies of the cellular ecosystem of childhood ALL will hopefully increase our understanding of mechanisms allowing the malignant cells to evade the immune system.

Single cell sequencing identifies distinct CD8 and CD4 T cell dynamics following anti-PDL1 and/or CD40 agonist in a murine model of pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) is the 4th leading cause of cancer related deaths with a dismal prognosis. Identifying a combination immunotherapy that has durable benefit for PDA patient treatment has been elusive. Here, using an orthotopic PDA mouse model that has a tractable endogenous antigen-specific T cell response, we employ single cell RNA sequencing (scRNAseq) + VDJ sequencing to understand the evolution of antitumor T cells and potential limitations of a combination immunotherapy that includes CD40 agonist + anti-PDL1. Following tumor establishment on day 7, 4 cohorts of mice were treated: 1) isotype, 2) anti-PDL1, 3) CD40

agonist, or 4) anti-PDL1 + CD40 agonist. On day 14, we isolated splenocytes and intratumoral immune cells and performed CITE-Seq, scRNAseq and VDJ sequencing using the 10X Genomics platform. CD40 agonist led to a reduction in naïve T cells and a concomitant expansion of *Cxcr3*+ CD4 and CD8 effector T cells in the spleen. Further, a terminally differentiated CD8 effector population with increased clonality and expressing *Cx3cr1* and *Klrg1* was uniquely expanded by CD40 agonist in the spleen. TCR sequencing showed that both CD4 and CD8 effector T cell populations traffic to the tumor, and the second most abundant TCRs from the terminally differentiated CD8 cluster in the spleen is the most abundant TCR in the tumor of anti-PDL1 + CD40 agonist treated mice. Cloning and expression of the most abundant intratumoral TCRs exhibit specific binding to a peptide:MHC tetramer representing a defined target tumor antigen. In the tumor, anti-PDL1 markedly enriched for clonally expanded T cells expressing *Cxcr3*, *S1pr1*, and *Ccl5*, suggesting these are recent T cell immigrants with the potential to recruit Ccr5+ T cells. In contrast, CD40 agonist expanded a population of putative stem like cells expressing *Sell*, *Tcf7*, *Id3*, and *Slamf6*, which in other infection and tumor models are thought to be critical for immunotherapy response. Lastly, CD40 agonist uniquely expanded intratumoral CD4 T cells that expressed *Cd40lg*, *Csf1*, and *Cxcr3*, suggesting these cells function to recruit and direct anti-tumor myeloid cells. Together, our results support that CD40 agonist promotes stem and effector T cell differentiation, while anti-PDL1 is necessary to direct the recruitment and clonal expansion of antitumor cells in the tumor bed. From these findings we hope to identify factors critical to immunotherapy response. Novel targets identified for enhancing immunotherapy efficacy for PDA patient treatment will be discussed.

Single-Cell Transcriptomic and Multiplex Imaging Analyses Reveal Both Overlapping and Distinct Mechanisms of Effective Immune Checkpoint Therapy and Neoantigen Cancer Vaccines

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Effective immunotherapy can induce profound tumor microenvironment (TME) remodeling, but this remodeling is incompletely understood. We sought to better define features of TME remodeling that are associated with responses to immunotherapy using models where we could differentiate between bystander and tumor antigen (Ag)-specific T cells. We first modified the YUMM1.7 (Y1.7) mouse melanoma line (that expresses endogenous non-mutant Ags) to additionally express defined MHC-I and MHC-II-point mutant neoantigens (NeoAgs). Whereas parental Y1.7 displayed aggressive outgrowth in mice treated with ICT, enforced expression of

an MHC-I and an MHC-II NeoAg rendered these Y1.7nAg melanomas sensitive to anti-PD-1 or anti-CTLA-4 immune checkpoint therapy (ICT). Interestingly, synthetic long peptide therapeutic NeoAg vaccines (NeoAgVax) induced Y1.7nAg tumor rejection that was comparable to ICT. We next assessed how different immunotherapies altered the TME using scRNAseq, flow cytometry, and CODEX imaging using a 42-marker panel. Anti-PD-1 and/or anti-CTLA-4 or NeoAgVax all induced an increase in conventional T cells, with NeoAgVax most dramatically amplifying highly functional tumor NeoAg-specific IFN-gamma+ CD8+ T cells, along with a concomitant reduction in the frequency of intratumoral M2-like macrophages. We also noted a dramatic therapy-induced increase in the frequency of intratumoral B cells, which have recently been implicated in response to ICT in patients. Whereas a majority of B cells induced by both ICT and NeoAgVax were phenotypically mature based on expression of Ms4a4c, Sell (CD62L), etc., these B cells could be further divided into several subpopulations with each treatment type inducing one or more distinct subpopulation of B cells, anti-PD-1 induced a subpopulation of B cells that differed from those induced by anti-CTLA-4, whereas the combination of anti-PD-1 and anti-CTLA-4 induced yet another B cell cluster that was distinct from those induced by either ICT used as monotherapy. Additionally, NeoAgVax induced a subpopulation of B cells that clustered separately from B cell subpopulations observed under control conditions or ICT. Finally, unlike tumors from control treated mice, CODEX imaging revealed tumors from mice undergoing ICT contained regions concentrated with CD4+ and CD8+ effector T cells and B cells expressing functional markers. Interestingly, these regions contained prominent blood vessels, along with MHC-II+ DCs and macrophages. Our work shows that immunotherapy reshapes the TME at the molecular and cellular level, including cellular localization within the TME, and that effective ICT leads to overlapping, as well as non-overlapping alterations when compared to NeoAgVax.

Spatially-Resolved Transcriptional Profiles of Macrophages are Prognostic in Diffuse Large B-cell Lymphoma

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Diffuse Large B-cell Lymphoma (DLBCL) is the most common haematological malignancy worldwide. DLBCL is treated upfront with R-CHOP combining anthracycline based chemotherapy and the monoclonal antibody Rituximab. ~40% of patients relapse after R-CHOP, and improving on this regimen requires understanding molecular determinants of poor

outcome. As the immune microenvironment plays a key role in determining clearance of cancer cells following therapy, we evaluated 30 immune-cell markers simultaneously in a tissue microarray of DLBCL samples (n=90) using the Nanostring Digital Spatial Profiling (DSP) platform. Amongst this range of immune features, CD163 (marker of tumour-associated macrophages (TAMs)) conferred the strongest negative prognostic effect.

Since the M1/M2 classification of TAMs does not capture the functional diversity of macrophage biology, we performed in-depth characterization of macrophages in the microenvironment of lymphoma and reactive lymphoid tissue (RLT) using spatial transcriptomics. We used the Nanostring GeoMx DSP platform to conduct a whole transcriptome analysis of cells selected by the pan-macrophage marker CD68 in a set of RLTs (n=17) and DLBCL samples (n=49). We uncovered diverse characteristics of macrophage transcriptomes in distinct spatial niches in lymphoid tissue; with clear distinctions between macrophages from the germinal center (GC) and interfollicular (IF) regions, as well as distinctions between macrophages in the light zone (LZ) and dark zone (DZ) of the GC. Macrophages from DLBCL tissue also differed significantly from those in RLTs, with further differences noted between cases with or without relapse. Based on these clusters of macrophage diversity we generated “spatially-derived macrophage signatures” (termed Macro-sigs).

These Macro-sigs associate with molecular subtypes of DLBCL based on cell-of-origin (COO). Across eight DLBCL gene expression datasets (totaling 4755 patients), the GC-like Macro-sig was enriched in germinal center B-cell (GCB) DLBCL, while the DLBCL Macro-sig was enriched in activated B-cell (ABC) DLBCL. Interestingly the IF-like Macro-sig was strongly enriched in cases categorized as “unclassified” for COO, raising the possibility that biological subtypes of DLBCL may evolve in parallel to their macrophage infiltration profile. In terms of prognostication, the relapsed-DLBCL macro-sig was indeed enriched in cases with shorter progression-free survival (n=4755, 8 cohorts). However, we unexpectedly noted that the most consistent association with poor survival after chemoimmunotherapy was with the DZ-like Macro-sig (significant in 8/8 cohorts). Using a previously published single-cell atlas of monocyte-macrophages (termed “MoMac-VERSE”), the DZ-like Macro-sig projects to a sub-cluster termed HES1/FOLR2 macrophages, considered to be tissue resident-like macrophages. Importantly the genes in the DZ-like Macro-sig are distinct from those in the relapse-like Macro-sig, and also distinct from the signature of DZ B-cells. Our work provides a foundation to evaluate mechanisms through which these DZ-like macrophages influence the microenvironment and tumour cell clearance after therapy, and identify potential therapeutic strategies to overcome their effect. Overall, these experiments provide the first spatially resolved transcriptomic analysis of macrophages in reactive and malignant lymphoid tissue, and identify novel macrophage sub-types with both biological and clinical significance.

Spatiotemporal Mapping of the Tumor-Draining Lymph Node in Melanoma

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Lymph node (LN) metastasis is a key negative prognostic factor in melanoma and other solid tumors. While the lymph node has historically been considered a passive conduit for metastasis, recent studies have illuminated the role of LN metastasis in promoting a state of systemic immunosuppression permissive of distant metastasis. However, the specific mechanisms activated by LN metastasis that suppress systemic immune surveillance are poorly understood. Here, we test the hypothesis that micrometastases in the tumor-draining LN (tdLN) install mechanisms of local and systemic immune suppression that permit further metastatic outgrowth. We use multimodal transcriptional data to describe the spatiotemporal remodeling of cellular networks in metastatic tdLNs in an inducible *Braf*^{V600E}*; Pten*$^{-/-}$ cutaneous melanoma. To quantitatively map the evolution of the tdLN we sampled naive and pre-metastatic (day 32), micrometastatic (day 64), and macrometastatic (day 98) tdLN and performed paired single cell RNA sequencing (scRNAseq) and spatial transcriptomics (ST). Unbiased clustering of scRNAseq data revealed heterogenous clusters of hematopoietic and non-hematopoietic cell types with both population level and transcriptional shifts as a function of disease progression. To dissect the spatial context of the tdLN, we performed unbiased clustering of the ST spots, which was sufficient to delineate the known structural compartments of the LN. Further, we integrated the scRNAseq and ST data by multimodal integration analysis and module score projections to map each unique cell population and substate to its tdLN location over time.

Preliminary analyses revealed an increase in regulatory T cells with LN metastasis, consistent with published human and murine studies. Additionally, we see evidence of both natural killer cell and CD8+ effector T cell activation, loss of cross-presenting *Batf3*$^{+}$ dendritic cells, and significant phenotypic shifts in resident B cell clusters. Deconvolution of spatial transcriptomic data revealed changing cellular neighborhoods at the tumor-immune interface, indicating potential mechanisms of cellular crosstalk critical for metastatic growth. Continued neighborhood analysis and functional studies will evaluate the effects of these changes on local and systemic metastasis. We predict that this unbiased and comprehensive understanding of the metastatic tdLN immune microenvironment will inform our model of metastatic progression

and reveal new strategies for LN reinvigoration, ultimately leading to improved systemic disease control.

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Spectra factor analysis deconvolves immunotherapy-induced gene programs from single-cell RNA sequencing data

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Our understanding of cellular biology is still largely based on discrete cell types. While single-cell RNA sequencing (scRNA-seq) has refined the granularity of known cell types, it is challenging to identify the continuous gene programs that underlie cellular states and processes. Factor analysis methods can identify such coherently expressed sets of genes ('gene programs') corresponding to biological pathways *de novo* using expression covariation, and pre-determined gene sets are also commonly used to identify gene programs from complex sequencing data. However, we lack an approach that combines the value of prior biological information with the discovery and contextual flexibility that unbiased analysis provides. Delineating gene program dynamics in scRNA-seq data from cancer patients will contribute a quantitative understanding of the transcriptional changes induced by cancer therapies.

We have developed Spectra, a factor analysis method that detects the activity of user-provided cell-type and gene program annotations, while adapting these programs to the biological context under analysis. Using a compact graph representation of gene programs, the algorithm selects a combination of *de novo* and known programs that best explain expression covariation, while explicitly separating cell-type-specific and global programs to control for levels of constitutive expression inherent to cell type. We apply Spectra to two scRNA-seq datasets from breast cancer patients treated with anti-PD-1 immune checkpoint inhibitors, and show that it outperforms existing factor analysis and gene set approaches in determining the effects of treatment on gene program dynamics in tumor infiltrating leukocytes.

Our analysis identifies known and novel features of tumor-reactive $CD8^+$ T cells—successfully separated from exhaustion states—which associate with clonal T cell expansion and include candidate immunotherapy targets. Spectra also reveals cell-type-specific metabolic gene programs and identifies lysine metabolism as a feature of tumor-infiltrating plasma cells linked to the unfolded protein response. Finally, Spectra finds cytokine and microenvironmental sensing programs that describe macrophage phenotypic plasticity under immune checkpoint therapy, in a continuous quantitative manner that goes beyond the unidimensional polarization (M1/M2) model. This includes a novel program of pro-metastatic macrophage features that is linked to immunotherapeutic resistance.

Spectra is thus a powerful method for deriving the gene programs underlying continuous cellular responses to immunotherapy, which can inform future experimental validation. Spectra will move single-cell analysis away from a reliance on cell aggregates tethered to discrete cell types, with possible implications for target and biomarker identification for cancer immunotherapies.

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Spontaneous T cell responses against cancer neoantigens

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Immunosurveillance refers to the ability of the immune system to detect and destroy malignant cells. While firmly established for virus-associated tumors, immunosurveillance of spontaneous tumors remains unclear. Previous autochthonous cancer models failed to resemble the sporadic nature of human cancer and involve at least one of the following drawbacks: no antigen known, surrogate antigen used, millions of cells simultaneously transformed, multiple tumors induced, early tolerance, tumor antigen(s) expressed in normal cells at the time of transformation, tumor antigen(s) expressed concomitant with viral infection. Cancer cell transplantation is artificial since it induces local acute inflammation that facilitates T cell activation and tumor rejection. We developed a transplantation model that overcomes this limitation by inoculating cancer cells in which antigenic oncogene expression and proliferation can be induced when the artificial transplantation-induced inflammation has subsided. This model involves the transplantation of cancer cells with doxycycline (dox)-inducible expression of the tumor antigen and tumor driver gene SV40 large T antigen (Tag) fused to luciferase (TagLuc).

Dox deprivation leads to growth arrest and loss of TagLuc expression in vitro. Following 2 weeks of dox deprivation in vitro, we subcutaneously inoculate cancer cells into immunocompetent and immunodeficient mice. After further 3 weeks, mice are administered dox to induce TagLuc expression and proliferation of cancer cells under non-inflammatory (resting) conditions. In vivo imaging revealed that while in immune-deficient mice tumors progressively grew, in T cell competent mice cancer cells were rejected. The loss of light signal occurred concomitant with the detection of antigen-specific CD8 T cells. Cancer cells were similarly rejected in visceral organs like liver, kidney capsule and the mammary gland. These results prove the robustness of our model to demonstrate immunosurveillance under resting conditions using a potent antigen with multiple MHC-I epitopes. However, most neoantigens result from a single amino acid exchange. Preliminary data with cancer cells expressing the neoantigen mutant p68 confirmed rejection if dox was never withdrawn (inflammatory conditions), but in sharp contrast to TagLuc, not anymore under resting conditions. These results suggest that CD8 T cells might have encountered mut-p68 but become dysfunctional. Overall, our model resembles pathophysiologic conditions allowing us to study the immune response to a single, slow-growing lesion starting from few cancer cells expressing clinically relevant antigens. Our results will provide a better understanding of the mechanisms underlying immunosurveillance or the inability of the immune system to control nascent tumors.

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STAPLER: efficient learning of TCR-pMHC recognition prediction from full-length αβ TCR-peptide data

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Prediction of the recognition of peptide-MHC (pMHC) complexes by αβ T-cell receptors (TCRs) remains challenging due to the extreme diversity of TCR and peptide sequences. To be able to

generalize to novel αβ TCR-peptide pairs, we hypothesized that a model should have a joint αβ TCR-peptide input, to allow the learning of patterns within and between TCR and peptide sequences that encode TCR-pMHC recognition. Importantly, as limited labeled αβ TCR-peptide data is currently available, the ability of such a model to efficiently learn from small amounts of data is critical. We developed STAPLER (Shared TCR And Peptide Language bidirectional Encoder Representations from transformers), a BERT model pre-trained on millions of unlabeled CDR3αβ-peptide pairs using the masked-language-modeling task, and fine-tuned on thousands of labeled full-length αβ TCR-peptide pairs. First, we show that data leakage during negative data generation can form a significant confounder. Next, we demonstrate that STAPLER outperforms prior models on predicting the recognition of seen peptides in a test dataset that does not contain this data leakage, in particular for peptides with little similar train data. Based on this ability to efficiently learn from limited labeled TCR-peptide data, we anticipate that STAPLER is best-suited to utilize growing TCR-pMHC datasets to reach better-than-random performance on predicting the recognition of unseen peptides.

STK11 Loss Promotes YAP1-Mediated Cytokine Expression in KRAS-Driven Lung Adenocarcinoma2012

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Serine Threonine Kinase 11 (STK11) loss of function (LoF) correlates clinically with resistance to anti-PD1 monoclonal antibody therapy in KRAS-driven non-small cell lung (NSCL) adenocarcinoma patients. Prior work suggests STK11 LoF leads to altered tumor-intrinsic cytokine expression profiles predicting an immunosuppressive tumor microenvironment (TME). However, the molecular mechanisms driving this observation remain unclear. To address this knowledge gap, we knocked out STK11 from three human KRAS-driven NSCL adenocarcinoma cell lines harboring wildtype STK11 alleles (NCI-H441, NCI-H1792, and NCI-H2009) and performed RNA sequencing to identify STK11-dependent transcriptome alterations potentially linked to anti-PD1 therapy resistance. Consistent with prior studies, our results revealed significant STK11-dependent changes in tumor intrinsic cytokine expression. Specifically, IL-6, IL-8, and CXCL2, all known to promote immunosuppression, demonstrated increased expression

in the absence of STK11 across all cell lines tested. Additionally, KEGG pathway analysis highlighted dysregulation of the HIPPO signaling network after STK11 ablation. Intriguingly, YAP1 activity, the ultimate transcriptional effector in the HIPPO network, has previously been linked with STK11, and subsequent Gene Set Enrichment Analysis identified an increased YAP1 signature in our STK11 knock-out cells. To evaluate the impact of YAP1 downstream of STK11, both genetic and pharmacologic inhibition of YAP1 were employed in our STK11 knock-out cells, resulting in reduced cytokine expression profiles comparable to cells with functional STK11. Expanding our work to Kras-driven and Kras-driven/Stk11-null NSCL adenocarcinoma mouse models confirmed Stk11-dependent alterations in cytokine expression and Yap1 activation in vivo. Additionally, serum from mice with Kras-driven/Stk11-null NSCL adenocarcinoma exhibited increased circulating IL-6 and CXCL1, the murine IL-8 homologue. These data support our hypothesis that tumor-intrinsic STK11 LoF is driving altered cytokine expression via a YAP1-mediated mechanism culminating in an immunosuppressive TME. We are now evaluating YAP1 as a potential therapeutic target to restore effectiveness of anti-PD1 therapy in Kras-driven NSCL adenocarcinomas lacking STK11.

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Successful Immune-Checkpoint Therapy Promotes a Functional Spatial Compartmentalization of the Tumor Microenvironment Organization

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The interactions between tumor, stroma and immune cells within the tumor microenvironment (TME) play key roles in determining tumor fate during cancer immunotherapy. We previously reported the use of high dimensional single cell profiling analyses (single cell RNASeq and Time-of-Flight Mass Cytometry) that characterized changes in intratumoral cellular populations in syngeneic mice bearing our well-characterized mouse T3 MCA sarcoma line in the presence or absence of immune checkpoint therapy (ICT). Whereas these studies clearly revealed the major remodeling of immune cell populations that correlated with successful ICT, they did not provide any spatial insights into the process. We therefore used CODEX multiplex imaging to compare the spatial interaction patterns occurring between key lymphoid and myeloid cell populations that result in either progressive T3 tumor outgrowth in mice treated with control antibody

(cmAb) or tumor rejection in mice treated with the combination of aPD-1 and aCTLA-4 (ICT). T3 tumor bearing mice were treated with cmAb or ICT, tumors were harvested on day10 (one day before ICT-induced rejection becomes detectable), fresh frozen, sectioned and then stained with a 35-antibody panel comprised of commercially available and custom-made CODEX antibodies. Large-field images encompassing the entire tumor section were subjected to image processing using an Akoya-developed pipeline. Unsupervised clustering based on normalized marker expression was used to profile 2,483,295 cells resulting in identification of 11 unique cell types. When compared to tumors from cmAb-treated mice, tumors from ICT-treated mice displayed a marked increase in the percentage of M1-like macrophages, Ly6G neutrophils and a corresponding decrease of M2-like macrophages. In addition, tumors from ICT-treated mice showed a significant decrease in CD140a+ tumor cells. Although the overall frequencies of CD4+ and CD8+ T cells in the ICT-treated versus cmAb-treated groups was not altered, the spatial proximity of the cells was much denser in ICT-treated mice than in cmAb-treated mice. Deeper spatial analysis of 5 distinct tumor compartments (stroma, tumor, surrounded-by-tumor, dead-tumor and tumor-front) revealed striking compartmentalization of tumor-associated immune cells after ICT treatment. For example, whereas tumors from cmAb-treated mice had similar levels of protein expression in all five compartments, tumors from αPD-1/αCTLA-4 treated mice displayed a distinct protein distribution pattern in each of the 5 compartments. Specifically, expression of Granzyme B and CD90 were found only in the surrounded-by-tumor compartment; expression of KI67, CD103, CD4 and CD8 were found only in the stroma compartment; cleaved caspase 3 and Ly6G were found exclusively in the dead-tumor compartment; and iNOS and PD-L1 expression were found at the tumor-front. Remodeling was also evidenced by results of cell localization analyses where, in tumors from cmAb-treated mice, CD4+ and CD8+ T cells were mostly located in the tumor compartment compared to their location in the stroma and surrounded-by-tumor compartments in tumors from ICT-treated mice. These results not only confirm our previously observed lymphoid and myeloid population remodeling observations but also reveal that in tumors destined to undergo successful ICT-induced rejection, changes in the spatial organization of immune cells drive a functional compartmentalization of the TME.

Surveillance of in situ tumor arrays reveals early environmental control of cancer immunity

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The immune phenotype of a tumor is a key predictor of its response to immunotherapy. Patients who respond to immune checkpoint blockade generally present with tumors infiltrated by T cells, a phenotype referred to as 'immune-inflamed'. However, not all inflamed tumors respond to therapy, and even lower response rates occur among patients with tumors that lack T cells ('immune-desert') or that spatially exclude T cells to the periphery of the tumor lesion ('immune-excluded'). Despite the importance of these tumor immune phenotypes in patients, little is known about their development, heterogeneity or dynamics due to the technical difficulty of tracking these features in situ.Here, we introduce STAMP (skin tumor array by microporation), a novel preclinical approach that combines in vivo high-throughput time-lapse imaging with next generation sequencing of tumor arrays. Using this approach, we follow the early formation of thousands of clonal tumors to show that the development of a given immune phenotype varies between adjacent tumors; it is not strictly determined by tumor genetics or systemic immunity but rather controlled by local features of the tumor microenvironment. The spatial distribution of T cells, specifically early T cell recruitment by fibroblasts and monocytes into the tumor core, was supportive of T cell cytotoxic activity and tumor rejection. Importantly, tumor immune phenotypes were not static over-time and an early conversion to the 'immune-inflamed' phenotype was predictive of therapy-induced or spontaneous tumor regression. Thus, STAMP captures the dynamic and complex relationships of spatial, cellular and molecular components of tumor rejection and has the potential to translate novel therapeutic concepts into successful clinical strategies.

(Minor revisions at Nature)

Systematic identification of cancer neoantigens derived from neo-open reading frames

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Immunotherapy has provided a major leap forward in the treatment of cancer. Major efforts focus on development of personalized neoantigen-directed immunotherapies, such as cancer vaccines and T-cell therapies. However, identification of highly immunogenic neoantigens as target for therapy from a tumor sample is challenging. Here, we integrate cancer whole genome and long-read transcript sequencing to identify the entire collection of novel open reading frame peptides (NOPs) expressed in tumors, termed the framome. As opposed to neoantigens derived from missense mutations, NOPs represent tumor-specific peptides that are completely different from wild-type proteins and thus have the potential to be strongly immunogenic. We also describe an uncharacterized class of hidden NOPs, which derive from structural genomic variants involving an upstream known protein coding gene that directly drives expression and translation of non-coding regions of the genome downstream of a rearrangement breakpoint. NOPs are prevalent in major cancer types and represent a vast amount of possible neoantigens particularly in tumors with many (complex) structural genomic variants and a low number of missense mutations. We show that epitopes derived from NOPs can bind to MHC class I molecules and we provide evidence for the presence of memory T-cells specific for hidden NOPs in lung cancer patient peripheral blood. Our work highlights a major source of neoantigens for personalized immunotherapies and provides a workflow for analyzing the complete cancer genome and transcriptome as a basis for systematic detection of NOPs.

T cell receptors targeting recurrent EGFR mutations in non-small cell lung cancer

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While most studies focus on the role of $CD8^+$ T cells that directly recognize tumor cells and mediate cell killing, $CD4^+$ T cells are required for the efficacy of immune checkpoint inhibition in murine models and the adoptive transfer of tumor-specific $CD4^+$ T cells without $CD8^+$ T cells can promote remissions in animal models and cancer patients. Recently, we and others have discovered transcriptional signatures of tumor antigen specific conventional $CD4^+$ T cells that are common across multiple solid tumor types, and the presence of cells with this signature correlates with clinical outcome after ICI treatment and with activation of other cells such as $CD8^+$ T cells, macrophages, and B cells. These observations suggest that tumor specific $CD4^+$ T cells have the ability to broadly shape the tumor microenvironment to enhance antitumor immunity. EGFR mutated lung adenocarcinoma clinically responds to kinase inhibitors but effective immune therapies in this subset are lacking, possibly due to a lack of mutations in a largely non-smoking population. EGFR mutant lung adenocarcinoma specifically lacks CD4+ T cells with this signature relative to EGFR unmutated tumors. We now describe the isolation of multiple CD4+ T cell clones specific to the recurrent EGFR L858R and exon 19 deletions from the peripheral blood of patients with these mutations. EGFR L858R specific T cell receptors (TCRs) are highly avid, mutation specific, and capable of recognizing processed antigen in MHC class II positive antigen presenting cells, including monocyte derived dendritic cells pulsed with material from EGFR L858R mutated tumor cells. We propose to adoptively transfer EGFR mutation specific TCR modified CD4+ T cells into patients to test whether increasing tumor specific CD4+ T cell responses within EGFR mutant lung cancer can activate the tumor microenvironment and mediate therapeutic benefit.

Targeting acquired resistance mutations in tumors using self-replicating RNA

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Despite the recent success of tumor immunotherapies, cancer vaccines have failed to translate into clinical efficacy. Apart from immunogenicity, selection of successful tumor antigen targets is confounded by clonal heterogeneity and removal of mutated clones via immune editing in both primary tumors and metastatic lesions. To address these issues, we have designed a novel approach to target acquired resistance mutations using self-replicating RNA (srRNA). In ER+ breast cancer, acquired resistance mutations develop in the ligand binding domain of the

estrogen receptor (ESR1) rendering them insensitive to endocrine therapies. Even in a heterogenous tumor environment, removal of cells harboring acquired resistance mutations will drive sensitivity of the remaining cells to concurrently administered standard-of-care endocrine therapy. This creates a lose-lose situation for individual tumor cells due to competing selective pressures: cells without acquired resistance mutations are removed via endocrine therapy, whereas mutated cells are removed by the immune system lengthening the time patients can effectively be on endocrine therapy. We have demonstrated that this srRNA vaccine can induce robust immune responses to targeted acquired resistance mutations and result in tumor control in mouse models. Based on these results, we will advance this candidate into clinical testing.

Targeting HSP90A sensitizes tumor to T cell-mediated therapy by reversing multi-modal resistance

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T cell-based immunotherapy has shifted the paradigm for cancer treatment. However, the majority of patients lack effective responses due to presence of immune-refractory tumor cells that limits its clinical success by blocking amplification of anti-tumor immunity cycle. Previously, we found that immune selection by T-cell based immunotherapy drives the evolution of tumors toward immunotherapeutic refractoriness including multimodal resistant and stem-like phenotype via activation of NANOG-TCL1A-AKT axis. Here, we report a crucial role of HSP90A at the crossroads between NANOG-TCL1A axis and the refractoriness of immune-refractory tumor cells by identifying HSP90AA1 as a novel NANOG transcriptional target. Furthermore, we found that TCL1A is a novel client of HSP90A, and demonstrated that HSP90A potentiates AKT activation through TCL1A stabilization and thereby contributes to refractoriness in $NANOG^{high}$ immune-refractory tumor cells. Importantly, inhibition of HSP90A sensitizes immune-refractory tumor cells to T cell-based immunotherapy and elicits effective anti-tumor response by re-invigorating anti-tumor immune cycle of tumor-reactive T cells. Our findings implicate that the NANOG-HSP90A pathway is a central molecular axis and a potential target for immune-refractory tumor.

TFF2-MSA Suppresses Tumor Growth and Increases Survival in an anti-PD-1 Treated MC38 Colorectal Cancer Model by Targeting MDSCs

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Myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment are a potential therapeutic target in immune checkpoint cancer therapy, but improved survival has yet to be shown targeting MDSCs. It has previously been demonstrated that trefoil factor family 2 (TFF2), a secreted anti-inflammatory peptide, can partially suppress MDSC expansion and partially activate tumor immunity through agonism of the CXCR4 receptor. We investigated whether a novel fusion protein, TFF2-murine serum albumin (TFF2-MSA), can improve survival in an anti-PD-1 treated syngeneic mouse colorectal cancer (CRC) model. We developed a model using MC38 CRC cells grafted subcutaneously into C57BL/6 mice. A recombinant protein, designated TFF2-MSA, which contains murine TFF2 fused to murine serum albumin (MSA), was generated with the goal of increasing half-life and reducing dose frequency. Mice subsequently received either TFF2-MSA, anti-PD-1 antibody (clone 29F.1A12), or both, and tumor volume, and survival were measured. Flow cytometry was performed to examine treatment-induced effects on immune profiles. Administration of TFF2-MSA suppressed tumor growth (TGI 38%), while the combination of TFF2-MSA and anti-PD-1 antibody had an additive effect and suppressed tumor growth dramatically (TGI 74%). Mice receiving both TFF2-MSA and anti-PD-1 exhibited a survival rate of 90% after 50 days, while vehicle and single TFF2-MSA therapy were 30% and 65%, respectively. The percentage of exhausted CD8+ T cells was markedly reduced in the draining lymph node by the combination treatment, as measured by flow cytometry using antibodies against LAG3, TIM3, and PD-1. TFF2-MSA in combination with checkpoint inhibition via anti-PD-1 antibody is additive in an advanced syngeneic mouse model of colorectal cancer.

The glycolipid-peptide conjugate vaccine induces antigen-specific CD8+ T cells that effectively inhibit the tumor cells that express human viral protein in mice

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Enhancing the proliferation and activation of antigen-specific CD8 T cells is one of the major goals of cancer vaccine development. In this study, we tested a synthetic glycolipid-peptide conjugate vaccine that consists of an E7 (43-77) synthetic long peptide from the HPV16 E7 oncogene and alpha-galactosylceramide (αGalCer), an iNKT agonist with vaccine adjuvant properties, covalently connected with a linker that incorporates proteolytic sites to release the peptide and αGalCer within the same antigen-presenting cell. A single dose of the therapeutic conjugate vaccine via tail vein injection induces a substantially higher number of E7-specific CD8 T cells *in vivo* and elicits significantly better tumor growth suppression than a mixture of its unconjugated components (admix of E7 peptide and αGalCer) in a model of E7-expressing lung cancer in mice in a type I NKT cell- and CD1d- dependent manner as tested in TCRJa18 and CD1d deficient mice, respectively. Moreover, this anti-tumor response depended on CD8+ T cells but not CD4+ T cells. In addition, the mice immunized with the conjugate vaccine had significantly fewer FoxP3+ Treg cells and plasmacytoid DCs in the tumor-infiltrating cells. Furthermore, the E7-αGalCer conjugate vaccine induced potent anti-tumor responses in both aged (20 months old) and young (2-3 months old) and male and female mice. Our data suggest that a synthetic glycolipid-peptide conjugate vaccine may prove useful for preventing or treating tumors expressing viral antigens.

The p53 suppression on tumor cells by SV40 T antigens and mutant p53 may recruit macrophages and modulate their expression of IDO and PD-L1

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The subtype of cervical cancer, for example, serous carcinoma and neuroendocrine tumor have p53 mutations. They progress and invade faster and metastasize faster and are more resistant to treatment of patients than other human papillomaviruses induced-cervical cancer having the wild-type p53. We found that Treg cells and tumor-associated macrophages were recruited at the invasive front of cervical cancer. These recruited macrophages are expressing IDO in cervical cancer patients (Cancer Sci 98:874-881 2007). In order to analyze the function of recruited macrophages at the invasive front area, we established αT3 mice carrying the αA-SV40Tag transgene, developing undifferentiated lens epithelial cancer in multistep

carcinogenesis (J Cancer Res Clin Oncol 135:1521-1532, 2009). The αT3 mice developed undifferentiated lens epithelial cancer similar to carcinogenesis pathologically as the high-risk human papillomavirus E6 and E7 oncoproteins-induced cervical cancer development. In this study, αT3 mice were mated with M1 mice expressing mutant p53 in lens cells (Proc Natl Acad Sci USA, 92:6142-6146, 1995), producing αT3M1 mice, and also mated with p53-deficient mice, producing p53-deficient αT3(αT3p53(-/-)) mice (Cancer Letters 179:165-173,2002, Oncology Report 12:253-258,2004). At 20 weeks of age of αT3, αT3M1 and αT3p53(-/-) mice, a proportion of progression to the invasive cancer was analyzed pathologically. And the number of recruited, IDO-expressing and PD-L1-expressing macrophages was determined by FACS analysis. The αT3M1 and αT3p53(-/-) tumors significantly progressed to invasive cancer more than αT3 tumors did. There were only a few macrophages in the wild-type lens, but there were more macrophages recruited in each lens tumor of αT3, αT3M1 and αT3p53(-/-). However, there was no significant difference in the number of IDO-expressing and PD-L1-expressing macrophages in the peritoneal cavity among wild-type, αT3, αT3M1 and αT3p53(-/-) mice. Moreover, we established tumor cell lines from each αT3, αT3M1 and αT3p53(-/-) tumor. We analyzed peritoneal macrophages after intraperitoneal cavity injection(i.p.) of these cells using wild-type mice. Both IDO-expressing and PD-L1-expressing peritoneal macrophages were significantly more recruited after each i.p. injection of αT3M1 and αTp53(-/-) cells than i.p. injection of αT3 cells. These results suggested that the amount of p53 suppression on tumor cells by SV40 T antigens and mutant p53 would be related to a number of both IDO-expressing and PD-L1-expressing recruited macrophages, which might suppress the local tumor immunity and enhance the invasion and progression of tumors. The PD-L1 expression on tumor cells is a good biomarker of immune checkpoint inhibiter. We will propose that the p53 mutation on tumor cells is also a good biomarker and that both PD-L1-expression and IDO-expression on macrophages at the area of the invasive and metastatic site are also biomarkers of the immune checkpoint inhibitors.

The RNA binding protein HuR is required for antigen presentation in tumor-T cells interactions

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Immunotherapies have shown limited effectiveness in pancreatic ductal adenocarcinoma (PDAC), despite successes in many other cancers. The RNA-binding protein HuR is known to play a critical role in the oncogenesis of PDAC through regulating key mRNA transcripts. Our lab has showed that tumor-intrinsic HuR regulates the tumor microenvironment composition and alters tumor cytokine release. Thus, we hypothesized that HuR plays a role in PDAC's interaction with the immune system. We disrupted the locus encoding HuR (*Elavl1*), using CRISPR Cas9 in a Kras-p53 mutant-driven (KPC) murine PDAC cell line to assess its function in tumor immune evasion. Here, we report that HuR is required for intact interferon responses and vulnerability to antitumor immune functions. Upon interferon-γ stimulation, HuR-deficient KPC cells failed to induce expression of MHC class I molecules, while PD-L1 levels were elevated as expected. Next, we transduced KPC cell lines with ovalbumin (OVA) antigen, which allowed us to selectively activate CD8+ OT-I T cells through their transgenic TCR. Consistent with previous findings, CD8+ OT-I T cells co-cultured with KPC OVA wildtype (WT) versus HuR-knockout (KO) demonstrated that the presence of HuR was necessary for effective T cell activation. Accordingly, in an orthotopic PDAC mouse model, HuR-proficiency in tumors was required for αPD-1 anti-tumor activity, as demonstrated by KPC HuR KO cells being resistant to αPD-1 treatment relative to KPC WT cells. Collectively, these data support the notion that HuR is required to mediate antigen presentation in PDAC tumor cells and therefore become vulnerable to CD8+ T cell mediated cytotoxicity. These findings support a paradigm shifting concept: HuR activation may be required for intact endogenous immune surveillance and responsiveness to immune-based anti-cancer therapies such as checkpoint blockade and T cell transfer. Future directions include deciphering the molecular mechanism behind this regulation, in hope to leveraging this function of HuR in improvement of PDAC immunotherapy.

The role of TCTP in controlling multi-malignant phenotypes and immune-resistance in immune-refractory tumor cells

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T cell-mediated immunotherapy has emerged as an effective therapy due to its durable responses. However, their benefits are only limited to few populations due to immunotherapeutic resistant tumors. Therefore, it is important to identify immunotherapy-resistant factors and develop clinically available drugs to reverse resistance and sensitizes T cell-mediated immunotherapy. Here, by establishing immunotherapy-refractory tumor models and

clinical transcriptome data on patients with cancer treated with anti-PD-L1 therapy, we identified TCTP as both the prognostic marker and targetable factor that confers multi-malignant phenotypes and immune-resistance in immune-refractory tumor cells. Moreover, we found that targeting TCTP using clinically available drug sensitizes tumors to T cell-mediated immunotherapy and reverses multi-malignant phenotypes of immune-refractory tumors. Thus, our study provides the rationale for targeting TCTP as an effective strategy to overcome resistance to T cell-mediated immunotherapy

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TIGIT and PD-L1 Co-blockade Promotes Functional Differentiation and Clonal Expansion of Anti-Tumor CD8+ T Cells Resistant to Exhaustion Programming

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Co-blockade of PD-L1 and TIGIT is an immunotherapy combination that has shown activity in both mouse tumor models and human cancer patients. In T cells, the two inhibitory pathways converge to regulate signaling by the costimulatory receptors CD28 and CD226. However, key questions have not been answered, such as whether the effects of the combination are taking place in the lymphoid tissue versus the tumor, and whether its action is on inhibiting versus reversing the differentiation of exhausted T cells. We examined how the combination of anti-TIGIT with anti-PD-L1 generates superior anti-tumor activity compared to either single agent alone by using a multi-omics approach in a mouse tumor model. Trafficking between tumor draining lymph nodes and tumor is required but only until sufficient tumor-specific $CD8^+$ T cells have infiltrated the tumor, at which point additional trafficking in no longer necessary. Combination treatment drives the dual expansion of tumor-specific $CD8^+$ T cells clones with a limited TCR repertoire in both draining lymph nodes and tumor, and these clones share phenotypic characteristics associated with stem cell memory-like or progenitors of exhausted cells that appear resistant to exhaustion programming. Furthermore, modification of the tumor-specific $CD8^+$ T cell profile is dependent on CD226 and an active Fc component of the anti-TIGIT antibody. We also find that a gene expression score representative of these dual expanded clones shows association with clinical benefit in a randomized phase 2 clinical trial evaluating tiragolumab (anti-TIGIT) plus atezolizumab (anti-PD-L1) in patients with non-small cell lung carcinoma. Thus, therapy combining Fc active anti-TIGIT with anti-PD-L1 promotes a tumor-specific $CD8^+$ T cell repertoire initially in lymphoid tissues that is qualitatively more effective at

mounting anti-tumor responses and also acts to reduce the differentiation of exhausted T cells. Dual blockade, therefore, may be effective by restricting entry into the exhaustion pathway, yielding expanded clonotypes that preferentially expand to effector and memory cells with a consequent potential for therapeutic benefit.

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Tiragolumab (anti-TIGIT) reverse translation reveals a myeloid cell-driven mechanism of action

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More than a dozen TIGIT antagonist antibodies are in clinical development. The most advanced anti-TIGIT, Tiragolumab, demonstrated significant clinical benefit when combined with Atezolizumab (anti-PD-L1) relative to treatment with Atezolizumab alone in the phase 2 NSCLC trial CITYSCAPE. However, there is no consensus on the mechanism(s) of action that are most relevant to anti-TIGIT treatment outcomes. In reverse translation analyses of CITYSCAPE and other patient specimens, we found that Tiragolumab leverages immunosuppressive macrophages and regulatory T cells (Treg) to remodel the tumor microenvironment and deliver therapeutic benefit. In mouse models, Tiragolumab surrogate antibodies drove antigen presentation and inflammatory gene programs in circulating and tumor-resident myeloid cells via activating Fc receptor engagement, resulting in downstream improvement to anti-tumor T cell responses. These data reveal a differentiated mechanism of action for TIGIT checkpoint

inhibitors and identify intratumoral myeloid cells and Fc receptor engagement as important considerations for patient benefit.

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Transcriptional hallmarks of persisting CD19 CAR T-cells in children with leukaemia

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Chimeric antigen receptor (CAR)-modified T-cells have become established as an effective treatment of haematological cancers. In the context of relapsed and refractory childhood pre-B cell acute lymphoblastic leukaemia (B ALL), CD19 targeting CAR T-cells often induce durable remissions. One of the most critical determinants of achieving a durable response is the persistence of CAR T-cells. Here, we systematically analysed CD19 CAR T cells of ten children with relapsed or refractory B ALL enrolled in the CARPALL trial (NCT02443831). We performed high throughput single-cell gene expression and T-cell receptor (TCR) sequencing of infusion products and serial blood and bone marrow samples up to five years post-infusion. Our key finding was that long lived CAR T cells developed a CD4/CD8 double-negative (DN) phenotype characterised by *GZMK*$^{+}$ exhausted-like memory state including expression of the co-inhibitory receptors *TIGIT* and *LAG3,* as well as the exhaustion regulator *TOX.* We observed the dominance of this distinctive persistence phenotype in all children with a long-lived treatment response. The phenotype emerged across clonotypes and subsets of T-cells, indicating that CAR T-cells converge transcriptionally when a durable clinical response is achieved. Remarkably, we also detected this persistence signature in recipients of a different CD19 CAR T-cell product that maintained decade long remissions in two adult patients with chronic lymphocytic leukaemia. Examination of single T-cell transcriptomes from a wide range of healthy and diseased tissues across children and adults indicated that the persistence signature is rarely encountered in other settings. Accordingly, we found a persistence signature that appears to be independent of infusion product, patient age, and leukaemia type. These findings raise the possibility that a universal transcriptional signature of clinically effective, persistent CD19 CAR T cells exist. It may provide a basis for the identification of biomarkers of persistence and guide refinement of manufacturing methods.

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TREM2-associated signature defines a tumor-enriched macrophage subset associated with response to checkpoint blockade in patients with hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) has a dismal prognosis, and though checkpoint blockade has significantly improved patient outcomes, many are left without clinical benefit, highlighting the need to identify additional immune targets to enhance therapeutic immunity. Macrophages (macs) are an abundant and heterogeneous population in the tumor microenvironment (TME), associated with poor prognosis in multiple tumor types, including HCC. However, their molecular and functional diversity is still poorly understood across tumor types. We analyzed the molecular and spatial organization patterns of immune cells within the TME and adjacent tissue of 35 resected HCC lesions using single-cell RNA sequencing (scRNAseq) and multiplex imaging. Our cohort is comprised of treatment-naïve patients, as well as responders and non-responders to neoadjuvant anti-PD-1 therapy (NCT03916627).

Using mass cytometry and scRNAseq of tumor and adjacent liver tissues, we mapped the molecular composition of macrophage programs during exposure to PD-1 blockade. Similar to our results in non-small cell lung cancer (NSCLC), we found that the self-renewing tissue-resident macrophages, also called Kupffer cells, were significantly depleted from the TME. In contrast, the TME was highly enriched in monocyte-derived macrophages (mo-macs), among which macs expressing high levels of *TREM2*, *GPNMB* and *CD9* (TREM2 macs) formed a significant population. Strikingly, we found that TREM2 macs were significantly enriched in responder patients compared to non-responders; they also accumulated in fibrotic areas and surrounding immune aggregates, in which they were found to interact with T cells. Surprisingly these results, in contrast to those that have shown that TREM2 macs dampen anti-tumor immunity in pre-clinical sarcoma and lung cancer models, including our own, prompted us to determine whether this enrichment for TREM2 macs in HCC patients responsive to PD-1 blockade could indeed be examined further.

Interestingly, the transcriptomic profile of TREM2 macs was largely conserved across human HCC and NSCLC lesions. In a pre-clinical model of HCC, we confirmed that analogous TREM2 macs were abundant in HCC lesions. Strikingly, TREM2 deficiency significantly reduced the survival of mice with HCC compared to wild-type (WT) mice. These results align with recent studies showing that TREM2 macs confer a protective effect against non-alcoholic fatty liver diseases (NAFLD), making the liver a particularly interesting organ to address the functionality of these macs.

Importantly, we also found that the level of soluble TREM2 (sTREM2), as measured in the blood of patients prior to treatment with PD-1 blockade, was significantly higher in that of responders compared to non-responders.

Altogether, these data reveal that the role of TREM2 macs in the anti-tumor response differs across tumor types, highlighting the importance of the tissue type in shaping immune cell function and caution against the universal use of TREM2 blockade in patients, especially in those with HCC. Importantly, these data also suggest that sTREM2 may be a biomarker of response to PD-1 blockade in HCC patients.

Triple synergy between cancer vaccine and checkpoint inhibitors in a pre-clinical tumor model

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Despite advances in checkpoint inhibitor (CPI) therapy for cancer treatment, many cancers remain resistant. Tumors deemed ‘cold’ based on lack of T cell infiltration into tumor stroma or low affinity show reduced potential for CPI therapy. Cancer vaccines may overcome this resistance by inducing the needed T cell immune response against the tumor to synergize with CPIs when the absence or low levels of anti-tumor T cells contributes to the primary resistance to CPIs. Here we used a mouse tumor model, TC1, that expresses HPV16 E6 and E7 oncogenes, and administered a vaccine consisting of the E7 synthetic long peptide combined with alpha-galactosylceramide (a potent NKT cell agonist) and GM-CSF as adjuvants. We show the synergy between the tumor-antigen specific vaccine and the combination of two CPIs, anti-TIGIT and anti-PD-L1. The synergistic effect of the triple combination provides more protection against tumor growth than either treatment alone or any pairwise combination and significantly

improves survival in a CD8+ T cell-dependent manner. Depletion of CD4 T cells surprisingly improved the vaccine response, and depleting FoxP3+ Tregs via diphtheria toxin in FoxP3-GFP^{DTR} mice revealed Tregs to be the causative agent inhibiting the response. The triple combination induces E7-specific T cells infiltrating the tumors by tetramer staining in young and aged mice, although aged mice show less protection than their younger counterparts. These data show proof-of-concept for a novel combination of a vaccine designed to elicit *de novo* anti-tumor T cell responses that can be amplified by synergistic CPIs and Treg depletion that lead to greater survival.

Tumor cell-intrinsic activity of gasdermin E contributes to checkpoint inhibitor-mediated anticancer immunity

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Introduction:

Durable clinical responses to immune checkpoint inhibitors are limited to a minority of patients, and molecular pathways that modulate their efficacy remain incompletely defined. We have recently shown that apoptosis in response to activation of innate nucleic acid receptors such as RIG-I facilitates tumor immunosurveillance and subsequent therapy. However, the molecular components that drive the immunogenicity of such apoptotic cells are unclear. We here show that intratumoral activity of gasdermin E (GSDME) links apoptotic to inflammatory cell death, thereby driving immunogenicity of tumor cell death and responsiveness to immune checkpoint inhibitors in murine melanoma.

Methods:

We used CRISPR/Cas9-edited melanoma and colorectal cancer cell lines (*Gsdme*$^{-/-}$) to address the role of GSDME in inflammatory cell death. We analyzed cell death induction in response to chemotherapy and nucleic acid receptor activation as well as tumor cell immunogenicity by

functional analysis of co-cultured dendritic cells. We studied the role of GSDME in a murine melanoma model in the context of immune checkpoint blockade. Furthermore, we retrospectively analyzed primary human melanoma samples at diagnosis and during checkpoint inhibitor treatment for the transcriptional activity of GSDME and its possible association with treatment outcome.

Results:

We found that induction of apoptosis in melanoma cells and colorectal cancer cells by activation of the RNA-sensing innate immune receptor RIG-I or chemotherapy treatment triggered cleavage of GSDME, with secondary loss of cell membrane integrity. This effect was significantly reduced in *Gsdme*$^{-/-}$ tumor cells. Morphologic analysis of tumor cells undergoing different forms of regulated cell death underlined the finding that GSDME can link apoptosis to inflammatory pyroptosis. In animals bearing *Gsdme*$^{-/-}$ tumors, the growth-delaying effect of anti-CTLA-4 and anti-PD-1 antibodies was markedly reduced, and the magnitude of the anti-tumor T-cell immune response was largely compromised. Analysis of tumor-draining lymph nodes showed that maturation and cross-presentation of tumor-associated antigens by dendritic cells was markedly reduce in mice bearing *Gsdme*$^{-/-}$ tumors. Similarly, in human melanoma cells, GSDME activity downstream of pro-apoptotic RIG-I signaling mediated a form of inflammatory cell death. RNA-Seq analysis of primary human melanoma samples did not reveal an association between intratumoral *Gsdme* expression and beneficial response to checkpoint inhibitor immunotherapy, suggesting that GSDME activity in tumors is not predominantly regulated on a transcriptional level.

Conclusions:

Our data show that secondary inflammatory cell death induced by intratumoral GSDME activity can facilitate immunogenicity of initially apoptotic cell death, and thereby contribute to the efficacy of checkpoint inhibitor therapy.

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Tumor-reactive CD4 Th cells co-express PD-1 and ICOS in human solid tumors

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CD4 T helper (Th) cells play a key role in orchestrating immune responses, but the identity of the CD4 Th cells involved in the immune response against cancer remains to be defined. To better define the composition of the CD4 Th cells infiltrating human solid tumors, we analyzed the phenotype of CD4 T cells in 22 patients with head and neck squamous cell carcinoma and 16 patients with microsatellite stable colorectal cancer by high dimensional flow cytometry. In addition, we determined their spatial location and cellular interactions in the tumor microenvironment by multiplex IHC to understand their role in the anti-tumor immune response. We also assessed the capacity of the CD4 Th cell populations sorted and expanded based on the expression of PD-1 and ICOS to recognize tumor-associated antigens and tumor-specific neoantigens. Finally, we investigated whether the presence of PD-1+ICOS+ CD4 Th cells in the tumor was associated with disease-free survival in HNSCC patients. Following t-SNE analysis, we identified a subset of CD4 Th cells distinct from FOXP3+ regulatory T cells that co-expressed PD-1 and ICOS. This cell population, which was present in the tumor but absent in the periphery, exhibited features of chronic stimulation and displayed characteristics of tissue resident memory T cells. PD-1+ICOS+ tumor-infiltrating (TIL) CD4 Th cells were located primarily in the tumor stroma in proximity to MHC class II+ cells and were proliferating, suggesting local antigen recognition. Interestingly, both PD-1+ICOS+ CD4 Th cells and CD39+CD103+ tumor-reactive CD8 T cells were enriched for cells secreting CXCL13, a chemokine involved in the recruitment of B cells. PD-1+ICOS+ CD4 Th TIL recognized tumor-associated antigens and also tumor-specific neoantigens, which were distinct from the epitopes recognized by the CD8 T cells from the same patients. Finally, higher frequencies of PD-1+ICOS+ CD4 Th TIL in patients with HNSCC was associated with better disease-free survival. Our findings provide an approach for isolating tumor-reactive CD4 Th TIL directly ex vivo that will help define their role in the anti-tumor immune response and potentially improve future adoptive T -cell therapy approaches.

Tumor-specific CD4 T cells instruct monocyte differentiation in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) is a lethal malignancy resistant to immunotherapy. PDA creates a suppressive fibroinflammatory tumor microenvironment (TME) composed of stromal and immune cells that inhibit anti-tumor immune responses. Tumor associated macrophages (TAMs) account for a large percentage of the TME and have remarkably heterogeneous functions, however, little is known about the local factors that drive monocyte differentiation into either pro- or anti-tumor states. Here, using a newly designed monocyte tracking mouse (CCR2CreER x R26tdTomato) implanted with orthotopic PDA tumors that express the click beetle luciferase (CB) neoantigen, we temporally tracked monocyte differentiation within tumors into TAM populations. We found that monocytes differentiate either into $MHCII^{hi}$ antitumor or $MHCII^{lo}$ Arginase-1 expressing protumor TAMs. We also identified a population of TAMs within PDA tumors that were derived independently of monocytes, but rather came from embryonic precursors and that displayed a CD206+ Folr2+ suppressive phenotype. Using antibody depletion and selective deletion of tumor specific CD4 T-cells, we found that CD4 T cells drive monocyte differentiation toward an antitumor TAM state. CD4 T cell depletion led to exacerbated tumor growth and monocyte-derived TAMs adopting a phenotypic and transcriptional state mirroring immunosuppressive pancreas tissue-resident macrophages. Mechanistically, monocyte acquisition of an antitumor TAM state was dependent on nonredundant IFNγ and CD40 signaling pathways. Loss of these pathways led to increased tumor growth. Finally, trajectory analyses and flow cytometric studies of PDA patient samples were consistent with a model in which circulating monocytes infiltrate the TME and can adopt a protumor or antitumor state, the latter potentially driven by CD40 signaling. Together these data are the first to address monocyte differentiation within PDA and identify previously unexpected role for CD4 T cells governing TAM differentiation program.

Uncovering the GPCR Landscape of the Tumor Infiltrating Lymphocytes

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G protein-coupled receptors (GPCRs) are the largest gene family of cell membrane-associated molecules mediating signal transmission, and their involvement in key physiological functions is well-established. All GPCRs exhibit a 7-transmembrane domain structure. Dysfunction of GPCRs contributes to some of the most prevalent human diseases, which is reflected by the 475 currently approved drugs that target 108 unique GPCRs and represent 34% of all FDA-approved drugs. However, GPCRs have been largely underexploited in oncology, and specifically underutilized in immune oncology. The importance and targetability of tumor infiltrating lymphocytes has been highlighted by the approval and success of immune checkpoint blockade (ICB) therapies for multiple cancer types. However, in general only a minority of patients respond to ICB. This raises the possibility that additional immunosuppressive mechanisms impacting tumor infiltrating lymphocytes might exist, which may represent novel therapeutic targets. Here, we mapped the expression patterns of all human GPCRs in 606,123 tumor infiltrating lymphocytes and myeloid cells in samples from 334 patients that span 26 different cancer types using a large-scale single cell RNAseq integration and analysis pipeline, thereby providing a multi-cancer resource for exploring GPCR biology in tumor infiltrating immune cells. We show that many GPCRs exhibit cell type specific expression across major immune cell subsets, and that some tumor infiltrating lymphocytes subsets express GPCRs that preferentially couple to particular G proteins families. Further, we define the druggable human immune onco-GPCRome, highlighting GPCRs expressed in specific tumor infiltrating immune cell subsets. These include GPCRs highly represented in immunostimulatory cytotoxic CD8 T cells, Treg CD4 T cells, and immune stimulatory and immune suppressive macrophage populations, which can be targeted by currently available drugs. This study provides a valuable resource to explore the role of GPCRs in cancer immunology. Ultimately, our study may reveal new and exciting GPCR therapeutic targets to enhance the response to currently available immune oncology agents, as well as help identify potential co-medications that may limit the success of ICB therapies.

Universal tumor targeting with CAR T cells specific for conjugated biotin

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Chimeric antigen receptor (CAR) T cells have significantly advanced the fight against B cell malignancies. Despite the success of CAR T cell therapies, high relapse rates, in part due to

antigen loss and T cell exhaustion, and toxicities have limited the use of CAR T cells in complex and antigenically diverse environments, such as solid tumors. Universal CAR platforms, which target tumors via bifunctional intermediate adaptors, simultaneously address these limitations to traditional CAR T cell therapies. The short half-life of intermediates and the modularity of their tumor-targeting moiety enable clinicians or researchers to address antigen loss, T cell exhaustion, and toxicities by controlling which tumor antigens are targeted, when CAR T cells have antigen presented, and how much antigen is presented. Here, we report the development of novel universal CAR T cell receptors that selectively target conjugated derivatives of biotin.

Through these studies, anti-conjugated biotin CAR T cells were tested for both target specificity and function. The CAR T cells were co-incubated with a biotin-Cy5 conjugate and titrated doses of either a non-fluorescent conjugated-biotin competitor or unmodified-biotin. Unlike the conjugated competitor, unmodified biotin had no impact on CAR binding to the soluble antigen, suggesting there will be no loss of function from these CAR T cells in the presence of endogenous or supplemented biotin in the body. The selectivity for conjugated biotin is a defining characteristic of this CAR. Intracellular cytokine staining and Meso Scale Discovery Assays demonstrated high cytokine production and release by the CAR T cells in the presence of target cells labeled with various conjugated-biotin intermediates. Likewise, chromium release assays and IncuCyte cell killing assays demonstrated effective killing of those same targets by the CAR T cells. These data collectively demonstrate the *in vitro* functionality of a new universal CAR platform to specifically target conjugated derivatives of biotin. These CARs have the potential to expand the repertoire of universal CARs and be used in combination with other universal CAR systems, such as an anti-FITC CAR. Pairing two universal CAR systems in the same therapy opens new possibilities to further reduce toxicity and T cell exhaustion through combination or logic-gated tumor targeting. The introduction of a new universal CAR allows for new, creative therapy strategies.

Vascularizing and immunizing cancer organoids with an assembloid approach assesses the efficacy of “do-not-eat-me” signal blockade in cancer-killing by macrophages

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The precise modeling of the tumor immune microenvironment *in vitro* is necessary for a better understanding of immune cell function against cancer cells. However, the current cancer organoids lack both vasculatures and immune cells, making it difficult to assess how immune

cells target cancer cells. We hypothesized that vascularizing and immunizing cancer organoids would model the tumor immune microenvironment precisely. We generated a three-dimensional structure of blood vessels and immune cells from human pluripotent stem cells. We induced embryoid bodies into hemato-endothelial lineage through morphogens and cytokines. We confirmed the generation of $CD34^+$ $CD45^+$ hematopoietic progenitor cells as well as $CD31^+$ endothelial cells. The resultant vascular-immune organoids (VIOs) were fused with the spheroid of hepatocellular carcinoma cell line HepG2, forming the assembloids. We revealed the thorough vascularization and immunization in entire assembloids. The imaging analyses identified the interaction between macrophages and cancer cells and further flow cytometry analysis confirmed phagocytosis of cancer cells. The assembloids offer a platform to model the blockade of “do-not-eat-me” signals by an anti-CD47 antibody to enhance macrophage phagocytosis. Altogether, we built assembloids that model the tumor immune microenvironment *in vitro*. We anticipate that the pre-clinical assessment of cancer immunotherapy will be boosted by the assembloids approach.